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FUNCTIONAL CHARACTERIZATION OF THE SPLIT SET AND MYND DOMAIN-CONTAINING METHYLTRANSFERASES, SMYD2 & SMYD3

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by

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Cell proliferation and differentiation are coordinated by synchronized patterns of gene expression. The regulation of these patterns is achieved, in part, through epigenetic mechanisms that affect the nature of DNA packaging into chromatin. Specifically, post-translational modifications to histone tails impact the structural dynamics of nucleosomes, thereby affecting DNA accessibility to transcriptional complexes. Accumulating evidence suggests that transcriptional regulators facilitate these alterations, resulting in altered local gene transcription. Thus, the structural interpretations of histone modifications are responsible for the establishment and maintenance of discrete programs of gene expression that ultimately correspond with distinct biological outcomes.

Most histone lysine methyltransferases catalyze methyl transfer by way of the SET domain, a module encoded within many proteins that regulate diverse processes, including some critical for development and proper progression of the

cell cycle. One such group of proteins, the **S**ET and **MY**N**D** domain (Smyd) family have been demonstrated to be direct regulators of tumorigenesis and essential developmental processes. Presented here is a functional characterization of two members of that family, Smyd2 and Smyd3.

Smyd2 is identified as a member of the Smyd family and reported here to possess SET-dependent histone H3, lysine 36-specific methyltransferase activity. Smyd2 specifically associates with the Sin3A histone deacetylase complex, suggesting a link between two independent chromatin modification activities. Finally, over-expression of Smyd2 in fibroblasts is shown to significantly suppress their rate of growth. It is therefore proposed that Smyd2-mediated chromatin modifications regulate specific gene expression, thereby having important implications for normal and neoplastic cell proliferation.

Aberrant expression of the histone H3-lysine 4-specific methyltransferase, Smyd3, has been implicated in colorectal, hepatocellular, and breast cell carcinogenesis. Here, Smyd3 is also shown to target histone H4, lysine 20 (H4K20). However, over-expression of Smyd3 in fibroblasts results in global reduction of trimethylation at H4K20 and this is accompanied by a striking increase in cell proliferation. As the methylation of H3K4 and H4K20 are normally associated with conflicting biological functions, I predict that these differential activities of Smyd3 are manifest under spatially and/or temporally distinct conditions, in the presence of different associating complexes, thereby resulting in effects that may be antagonistic of one another.

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1. INTRODUCTION

1.1 Background

1.11 Chromatin

From regulated gene expression to mitosis, chromatin acts as a structurally flexible repository of the genome [1]. In this manifestation, an entire chromosome is sequentially compacted through a series of highly ordered packaging while distinct regions of DNA are selectively made accessible to transcriptional complexes. Thus, chromatin maintains a dynamic architecture that allows approximately 2 m of DNA to be parceled in the nucleus while retaining a remarkable degree of functionality (Fig. 1.1) [2, 3].

At its foundation, chromatin is grounded in a succession of nucleosomes, the basic structural units [4], consisting of 146 base pairs of DNA, wrapped 1.7 times around an octamer of core histones and separated by a linker region of approximately 50 base pairs. The primary histones involved in the assembly of a nucleosome are histones H2A, H2B, H3 and H4. These histones ultimately form octamers, such that each is represented twice in the nucleosome core unit [5].

The structure of each histone is highly conserved, including a folded core and an unstructured tail [6]. The histone core is a globular domain, forming a helix-turn-helix motif, which facilitates dimerization. Conversely, the tails of histones do not adopt defined conformations, as demonstrated in x-ray crystallographic studies, except when bound to their cognate proteins [6]. These tail domains contain a number of conserved amino acid residues including lysine,

arginine, and serine [7]. Histone tails, which sustain a basic charge, can interact with the poly-anionic backbone of the core DNA, marginally contributing to nucleosomal stability [8]. Therefore, regulation of chromatin structure and transcription is often mediated through post-translational modifications that alter specific residues along these tails. These modifications can affect the accessibility of nuclear factors to DNA or induce the recruitment of such factors to transcriptional or chromatin assembly pathways [9].

Histone-DNA interactions are formed primarily by rigid hydrogen bonds between the histone main chain amide and the phosphate oxygen of the DNA. These are strengthened by electrostatic interactions between basic side chains and negatively charged phosphate groups and other nonpolar interactions [10]. While this allows, in theory, nucleosome formation on any DNA sequence, there may be specific sequence preferences for nucleosomal positioning [11]. The nature of the underlying DNA sequences, by which the histone core is wrapped, is likely the major determinant of the core histone displacement and the dynamic behavior of the nucleosome under the influence of the SWI/SNF ATPase and sequence-specific transcription factors [12]. The most thoroughly characterized nucleosomal assembly is the 30nm fiber, which is stabilized by linker histones [13-15] and the relative positioning of each nucleosome [16], ensuring intimate physical proximity while producing minimal internucleosomal attraction energy [17, 18]. Thus, this structure allows dramatic changes in the degree of compaction to occur without a concomitant change in topology.

Chromatin is manifest in a number of additionally heightened states of compaction [19], and even higher order structures occur upon interaction with non-histone, architectural proteins [20]. In the past three decades, a number of chromatin-related events including DNA methylation, incorporation of histone variants, post-translational modifications of histones, and ATP-dependent chromatin remodeling have been intensely studied. These modifications, and the protein complexes involved with their facilitation, have been linked to the regulation of many biological processes dependent upon the accessibility of chromatin [21-24]. These include gene expression, DNA repair, chromosome segregation during mitosis, X chromosome inactivation, and chromatin condensation during apoptosis [25-28].

Chromatin modifications impart epigenetic control of gene expression without requisite changes in DNA sequence. Disrupting the balance of epigenetic networks has been linked to severe pathological consequences, including tumorigenesis, syndromes involving chromosomal instability, and neurological disorders [29-31]. Recent advances in our understanding of chromatin structure/regulation and epigenetic inheritance have led to the development of promising new therapies that target the enzymes and complexes that are responsible [32].

1.12 DNA accessibility in transcriptional regulation

The structure of heterochromatin restricts physical access of nuclear factors to the underlying DNA [33]. Regulation of chromatin architecture is, therefore, necessary but not sufficient for controlling gene expression. The involvement of sequence-specific activators, repressors, mediator complexes, and general transcription factors are also required to manage transcriptional regulation [34, 35]. During transcriptional activation, the binding of gene-specific factors to defined DNA sequences triggers a cascade of spatially and temporally coordinated reactions. These result in a chromatin template, appropriately remodeled, which enhances the binding of ubiquitous transcription factors and the general transcription machinery [36, 37].

Transcription factors interact with specific sequences and are divided into three classifications. General transcription factors are subunits of the RNA Polymerase II complex, which transcribes template DNA into messenger RNA [38]. The upstream regulatory transcription factors recognize consensus elements located in promoter regions and act by increasing the efficiency of transcription initiation. General transcription factors and upstream transcription factors are ubiquitous factors that require accessible chromatin structure for DNA binding [38]. This is accomplished by the third group of transcription factors which induce the structural remodeling required to open distinct regions of chromatin. These inducible factors are gene-specific and are synthesized or activated at discrete times and in distinct tissues. For example, nuclear receptors, which constitute a large family of ligand-inducible transcription factors,

have the capacity to bind to condensed chromatin templates [39]. The response of a given receptor to a particular ligand depends on the set of co-regulators with which it is able to interact. Recruited co-regulators are able to covalently modify histones or remodel nucleosomes in an ATP-dependent manner and these alterations modulate the promoter accessibility to both common transcription factors as well as the basal transcriptional machinery [40]. Ultimately, transcriptional activation results from the integration of specific and ubiquitous factor-binding at the promoter, suggesting that the constitution of the promoter is of critical importance [41].

1.13 ATP-dependent nucleosome remodeling

Nucleosomal remodeling is an ATP-dependent process that alters chromatin structure in a non-covalent manner [42]. The complexes that facilitate this process are of fundamental importance because they affect the accessibility of DNA to other complexes involved in transcription, DNA repair, and replication. Thus, ATP-dependent chromatin remodeling can affect gene expression, cell cycle progression, and cell differentiation [43]. Chromatin remodeling complexes are divided into several classes, based on variation within their catalytic ATPase subunits. Although these subunits display homology within the ATPase domain, additional domains vary significantly among classes. For example, the SWI/SNF family contains a bromo domain [44], the ISWI family contains a SANT domain [45], and the Mi-2/NURD family, a chromo domain [46]. Each ATPase

associates with different subunits to form distinct multiprotein complexes and each subunit may be differentially involved in the regulation or targeting of remodeling activity (Fig. 1.2) [47].

Nagaich and colleagues studied the interaction between the glucocorticoid receptor (GR) and an array of nucleosomes, assembled on the mouse mammary tumor virus long terminal repeat. They observed that receptor binding to nucleosomal DNA is enhanced by SWI/SNF and is accompanied by sequential reorganization of histone proteins within the nucleosomes. The action of SWI/SNF is proposed to lead to changes in the position of histone H2B within the nucleosome in concert with the recruitment of GR to a new binding site within the nucleosomal DNA [48]. Recent advances have allowed nucleosome dynamics on promoters to be studied in real time and support the idea that individual nucleosomes may have an inherent capacity to 'breathe' [49].

1.14 DNA methylation

Methylation of DNA is a covalent modification that occurs at cytosines within CpG-rich regions of DNA and is catalyzed by DNA methyltransferases [50]. The methylation of DNA affects the binding of proteins to their cognate DNA sequences [51]. Such addition of methyl groups can prevent the binding of basal transcriptional machinery and ubiquitous transcription factors [52]. In this way, DNA methylation contributes to epigenetic inheritance, allele-specific expression, inactivation of the X chromosome, genomic stability and embryonic

development [53]. Progressive DNA methylation is widely implicated as an agent of normal senescence as well as tumorigenesis [54].

The majority of methylated CpG islands are located within repetitive elements including centromeric repeats, satellite sequences, and gene duplications. These CpG regions are often found at the 5′ end of genes where DNA methylation affects transcription by recruiting methyl-CpG binding domain (MBD) proteins that function as adaptors between methylated DNA and chromatin-modifying enzymes [55]. There is a clear relationship between DNA methylation and other silencing mechanisms including histone modifications and chromatin remodeling [56, 57]. In fact, several studies suggest that DNA methylation is targeted specifically toward genes that are already suppressed by other mechanisms [54].

1.15 Histone modifications

Histone tail alterations encompass the greatest range of variation in epigenetic regulation, encompassing more than 50 known sites of modification [58]. Histones are subject to several forms of post-translational modification, including methylation, citrullination, acetylation, phosphorylation, SUMOylation and ADP-ribosylation (Fig. 1.3) [7, 59]. These modifications impart biological consequences by acting as marks for the specific recruitment of regulatory complexes and affecting the structure of the nucleosome. Acting in concert, the combination of different histone modifications is thought to constitute a “histone code” that is interpreted in the form of specific nuclear events [60, 61].

Although the interplay among various histone modifications is still largely nebulous, a paradigm is rapidly emerging whereby methylation, acetylation, or phosphorylation at independent sites may work in tandem with other such modifications to convey unique biological consequences [62]. Such crosstalk has already been clearly demonstrated by a number of findings including the cooperation between acetylation and phosphorylation of histone H3 during the cell cycle [63], the correlation between acetylation and arginine methylation in the regulation of estrogen-responsive genes [64], and the competition between methylation and acetylation of histone H3, lysine 9 toward the establishment or disruption of heterochromatin [65]. As new studies continue to highlight the importance of crosstalk in epigenetic regulation, our early understanding of singular histone modifications have yielded to a more delicate model in which minor variations in broad patterns of modifications impart distinct outcomes.

In 1964, Allfrey and colleagues noted a correlation between increased histone acetylation and augmented transcription [66]. Since then, much has been uncovered regarding the affects of histone acetylation. This modification has been implicated in DNA replication, DNA repair, and modulation of chromatin structure [67]. Hyper-acetylation of histone lysine residues is thought to influence transcriptional activity by neutralizing the positive charge of the histone tails and decreasing their affinity for negatively charged DNA, thereby allowing access of transcription factors to promoters [68-70]. Conversely, histone deacetylation is believed to hinder the accessibility of DNA by restoring the net positive charge [71]. In addition to charge-neutralization, more recent studies indicate that

histone acetylation/deacetylation regulates transcription by altering higher-order folding properties of the chromatin fiber and providing specific binding surfaces for the recruitment of transcription co-regulators [69].

Near promoter sites, acetylation of histone amino-termini provides binding surfaces for transcription factors of the TFIID transcription initiation complex as well as for proteins in chromatin-remodeling complexes [72]. Agalioti and colleagues have shown progressive acetylation of the human interferon (IFN)-β gene upon transcriptional activation. Each acetylation pattern correlated with the recruitment of a specific protein. The general transcription factors GCN5 and $TAF_{II}250$, the largest subunit of the TFIID complex, are recruited to target promoter regions and sequentially acetylate H4 lysine 8 and H3 lysine 9 and 14, respectively. In turn, H4 lysine 8 acetylation provides a binding site for BRG1 that is part of the SWI/SNF complex that promotes ATP-dependent nucleosome remodeling [72]. In addition to affecting chromatin dynamics through alteration of histone tails, recent studies indicate that acetylation of lysines at the edge of the histone globular domain is also possible and this modification facilitates the recruitment of chromatin remodeling complexes in yeast [73].

The first cloned histone acetyltransferase (HAT) was obtained from *Tetrahymena thermophilia* [74], and sequence similarity with previously identified transcription factors such as CBP/p300, $TAF_{II}250$, and SRC-1, revealed that these transcriptional co-activators all possessed HAT activity [67, 75]. These findings strengthen the idea that local acetylation of histones by transcription factors contributes to the activation of promoter-specific gene expression.

Histone acetylases function in large complexes, such that associating subunits can modulate HAT activity and substrate specificity. In addition, HAT activity can be affected by sequence-specific transcription factors as well as other histone modifications [76]. Homozygous deletions of distinct histone acetylases, *in vivo,* are manifest by disparate developmental defects, suggesting a highly specialized functionality for these enzymes [77].

Antagonism of HAT activity is achieved by a group of enzymes called histone deacetylases (HDACs). Traditionally, these are thought to impart transcriptional repression by catalyzing the removal of the acetyl moiety from histone lysines [76]. The first mammalian HDAC identified is related to the yeast transcriptional regulator, Rpd3 [78]. Since then, additional HDACs have been discovered and parceled into subclasses, based on sequence homology with their yeast homologs. The human class I histone deacetylases, similar to Rpd3, include HDACs 1, 2, 3 and 8. A second class, including HDACs 4, 5, 6, 7, 9 and 10, are similar to the yeast Hda1 and are regulated through subcellular localization. Class III HDACs, also referred to as the sirtuins, exhibit significant sequence and functional divergence from the class I and II groups [71]. This third class of HDACs displays NAD-dependent deacetylase activity, similar to the yeast Sir2 protein, and play an essential role in epigenetic silencing [79]. Uniquely, class III HDACs are not sensitive to traditional HDAC inhibitors such as trichostatin A or valproic acid. Although the substrate specificity of distinct HDACs remains ill-defined, phylogenetic analyses reveal that HDACs evolved in the absence of histone proteins, suggesting that primary HDAC substrates may

not be histones [76]. In addition to its classic role, invoking transcriptional repression, contemporary studies have revealed that deacetylation is also required at the promoters of many transcriptionally active genes [80]. Thus, histone deacetylation is an excellent example of the paradoxical complexities of the histone code.

While acetylation of histone tails is largely ephemeral in nature, histone methylation is widely observed to be a mark that confers long-standing epigenetic memory [81]. Mounting evidence suggests that histone lysine methylation is a critical factor in such pathways as transcriptional regulation, X chromosome inactivation, DNA methylation, and the formation of heterochromatin [25-27]. Catalyzed by histone methyltransferases, this modification ultimately mediates either gene activation or silencing, in a residue-dependent manner [81]. The level of specificity is heightened by the variation in biological consequences associated with whether a residue is mono-, di-, or tri-methylated [82, 83]. It has also been reported that many transient histone modifications work in tandem with histone lysine methylation, further increasing the potential complexity of this epigenetic modification [1].

Most histone lysine methyltransferases catalyze methyl transfer by way of the SET domain, a module encoded within many proteins that regulate diverse processes, including some critical for development and proper progression of the cell cycle [27, 60, 84]. Residue-specific histone lysine methylation typically correlates with distinct states of gene expression [85]. Most of the known

targeted lysines of histone methyltransferases occur on histone H3 which thereby serves as a conduit of epigenetic regulation. In general, lysine methylation at histone H3, lysine 9 (H3K9), H3K27, or H4K20 corresponds with gene silencing, whereas methylation of H3K4, H3K36, or H3K79 is associated with actively transcribed genes. However these paradigms are far too narrow to encompass the growing intricacies of the histone code [85]. Recent evidence implicates histone methylation in the recruitment of chromatin remodeling complexes, as is the case with CHD1, an ATP-dependent chromatin remodeling factor that specifically binds methylated H3K4 [86]. Once thought to be a permanent modification, enzymes have now been identified that are capable of reversing histone methylation at specific sites [81, 87].

The incorporation of histone variants provides yet another echelon to the capacity of epigenetic mechanisms to store cellular information [28]. Locally, this affects nucleosome structure as well as the propensity of variant-containing chromatin to be remodeled. Hence, histone variant incorporation can alter nucleosome stability, mobility, and potential patterns of histone modifications, likely affecting higher order structure and downstream events [88-90]. For example, a specialized H3-like variant CENP-A, replaces H3 in centromeric nucleosomes to maintain a unique structure that is critical for proper chromosomal segregation [91]. There are many present studies emphasizing the physiological relevance of histone variants and their growing role in our understanding of epigenetic regulation [28].

1.16 Non-coding RNA

Accumulating evidence suggests the existence of RNA regulatory networks that are involved in the regulation of gene expression at various levels [92]. It has been observed that non-coding RNA, targeting CpG islands in promoter regions, acts in concert with both DNA and histone methylation to affect gene transcription [93-95]. In fission yeast and in *Drosophila*, the involvement of small interfering RNA has been studied in sequence-specific targeting of transgenes, transposable elements, heterochromatin, and some cases of polycomb-mediated gene silencing [96]. Although the current understanding of the influence of non-coding RNA on transcriptional activity is still incomplete, this is an exciting new front in the field of epigenetic modifications that promises to possess answers to broader questions on transcriptional regulation [97].

1.2 Smyd Family Significance

The development of tissues and organisms depends upon the acquisition of distinct programs for gene expression among individual cell types [98]. These programs are maintained in a heritable state by epigenetic mechanisms that impart cellular memory [99]. In this way, the global synchronization of patterns in gene expression broadly dictates developmental consequences [100]. At the core of such gene regulation are mechanistic pathways that affect the packaging of DNA into chromatin, thereby establishing the degree of DNA accessibility to transcriptional complexes [58, 60, 100, 101]. These pathways are highlighted in

preceding sections and include DNA methylation, chromatin remodeling, histone replacement, and alterations to histone tails [60, 102, 103].

As described in section 1.15, most histone lysine methylation is catalyzed by proteins containing a SET domain [27, 60]. The **SET** and **MYND** (Smyd) family comprises a subset of five SET domain-containing proteins that have unique domain architecture. Specifically, this family of proteins is defined by a SET domain that is split into two segments by a MYND domain, followed by a cysteine-rich post-SET domain [104]. Members of this family may be important in developmental regulation, as targeted disruption of the Smyd1 gene results in impaired cardiomyocyte maturation, flawed cardiac morphogenesis, and embryonic lethality (Fig. 1.4) [104]. Functionally, Smyd1 is thought to regulate gene expression via its association with histone deacetylase activity [104] and the muscle-specific transcription factor, skNAC [104, 105]. Recent evidence demonstrates that skNAC is methylated by Smyd1 (personal communication from Dr. Li Zhu).

Pathological consequences have also been associated with aberrant expression of Smyd proteins in mature tissues. Smyd3 has been noted for its involvement in cancer cell proliferation, underscoring the importance of proper regulation of the Smyd family members. Smyd3 is over-expressed in most hepatocellular and colorectal carcinomas, and its exogenous over-expression in fibroblasts significantly augments growth. Functionally, Smyd3 has been shown to have an active SET domain that modulates chromatin structure through its intrinsic H3K4-specific histone lysine methyltransferase (HKMT) activity. The

enhancement of cell growth that is associated with over-expression of Smyd3 is completely abolished when the SET domain is mutated. [106]. Further investigation of the activity and specificity of the SET domain in other Smyd proteins is critical for our understanding of the broader functional and biological potential of this family.

The MYND domain of Smyd proteins encompasses a putative zinc-finger motif that facilitates protein-protein interactions. This domain is present in several other transcriptional regulators where it is known to contribute in developmental processes [107, 108]. Interface at the MYND domain occurs through a PXLXP motif in the associating protein. For example, the associations of Smyd1B with HDACs and the transcription factor, skNAC, are mediated through these sites [104, 105].

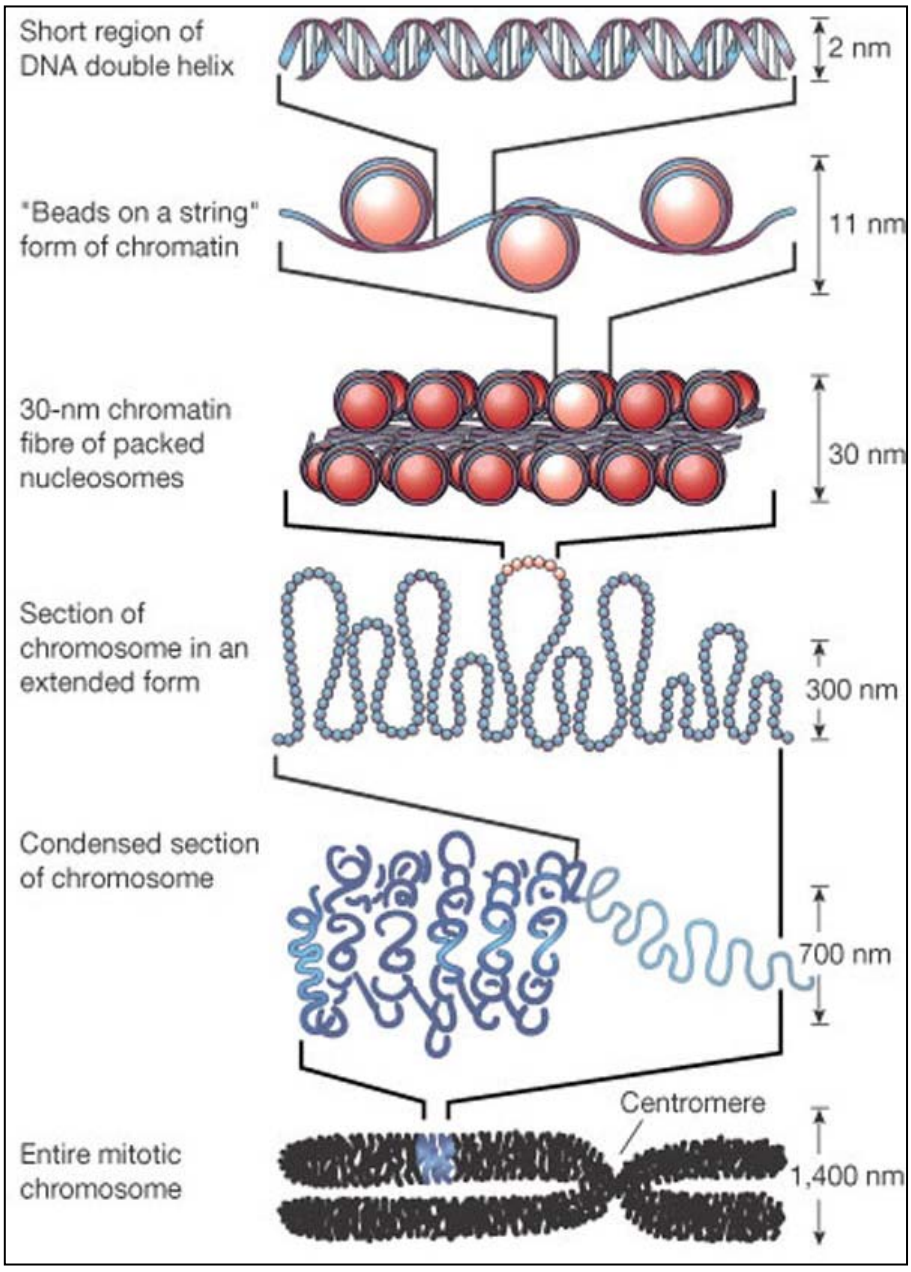

Figure 1.1: Chromatin condensation. The basic structural unit of chromatin is the nucleosome, in which double-helical DNA wraps around an octamer of core histones. Each unit is connected to the next nucleosome by a short sequence of linker DNA. This 'beads on a string' architecture is further condensed into the '30 nm fibre.' Chromatin is packed additionally into higher-order structures, allowing approximately 2 meters of cellular DNA to be parceled in the nucleus. (Adapted from Felsenfeld, et al. [3])

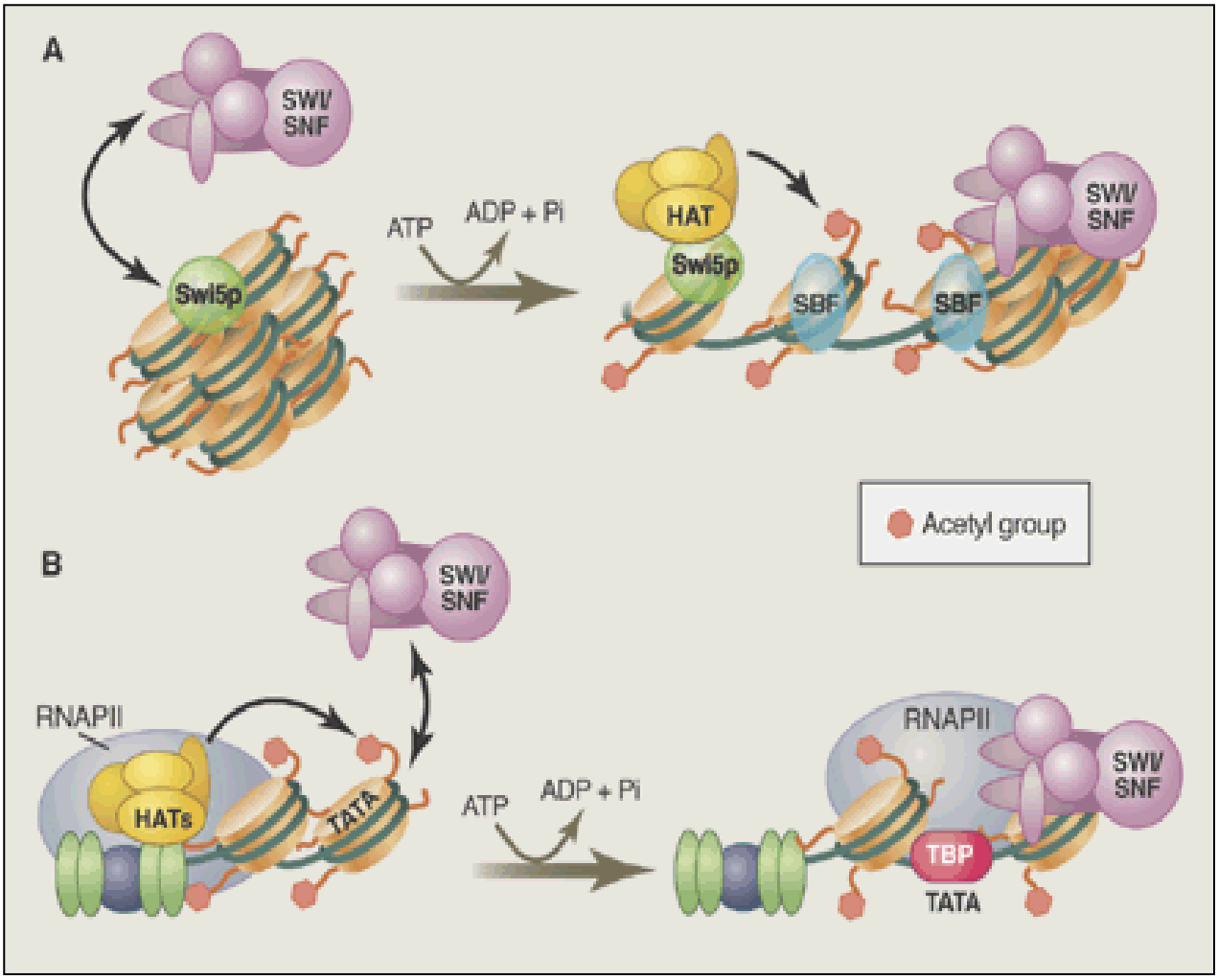

Figure 1.2: Remodeling chromatin. Chromatin-remodeling is required for transcriptional activation. **(**A**)** At the gene promoter, Swi5p recruits the SWI/SNF and HAT chromatin-remodeling complexes to initiate transcription. SWI/SNF and the HAT complex cooperatively facilitate binding of gene-specific activators such as SBF. **(**B**)** Upstream activating elements (blue and green) facilitate the recruitment of HATs during assembly of the RNA polymerase II (RNAPII) pre-initiation complex. Histone acetylation recruits the SWI/SNF complex, which further disrupts the structure of the promoter-bound nucleosome. This permits TBP binding to the TATA element, and completes assembly of the pre-initiation complex. (Adapted from Fry and Peterson [47])

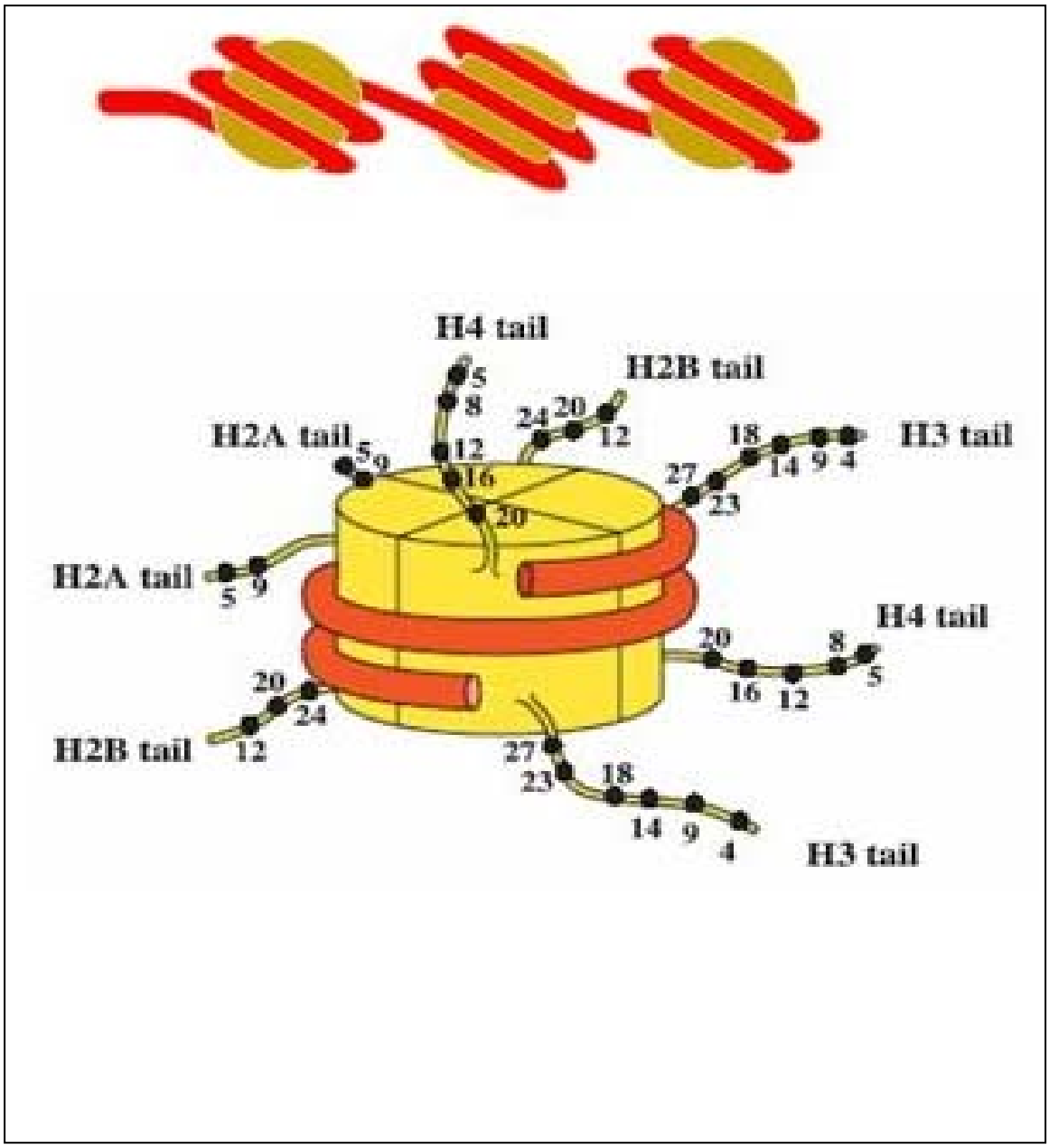

Figure 1.3: Common sites of histone modifications. Histone tail alterations encompass the greatest range of variation in epigenetic regulation. Shown here schematically, N-terminal tails project from within the histone core of chromatin. Numbers correspond to specific residues of the associated tails that are known to be the targets of a variety of post-translational modifications. These alterations impact the degree of chromatin condensation which, in turn, regulates transcriptional activity. (Adapted from Zheng [59])

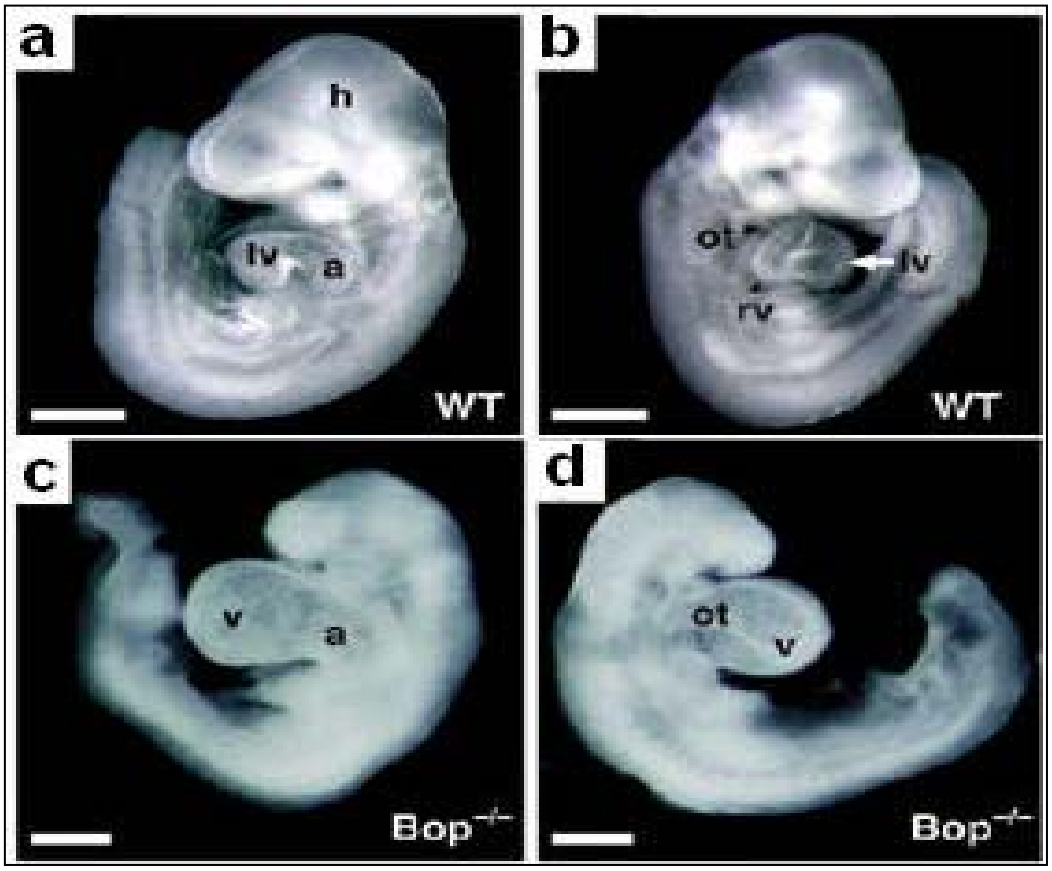

Figure 1.4 Targeted deletion of Smyd1 (Bop) results in embryonic lethality due to cardiac enlargement. Left and right lateral views of wildtype (WT; a,b) and Smyd mutant (c,d) embryos at E9.5 are shown. Gross analysis of mutant embryos shows growth retardation, an enlarged ventricular chamber (v) and a single ventricular segment, in contrast to wildtype embryos. Scale bars, 400 μm. **a**, atria; **h**, head; **lv**, left ventricle; **ot**, outflow tract; **rv**, right ventricle. (Adapted from Gottlieb, et al. [104])

2. MATERIALS AND METHODS

2.1 Computational analysis

The Smyd family members were identified from BLAST comparisons using the protein databases found at the National Center for Biotechnology Information (NCBI) web site (http://www.ncbi.nlm.nih.gov/). The ClustalW and BOXSHADE programs were used for alignments and shading of Smyd family proteins

2.2 Cell culture

Cells lines were grown in DMEM, supplemented with 10% fetal bovine serum, 1 mM L-glutamine, 1% non-essential amino acids, penicillin, streptomycin, and fungizone (all from Life Technologies), at 37° C in a humidified atmosphere of 5% CO_2. 293T, 10T1/2, HeLa, and NIH3T3 cells, obtained from ATCC, were used in these studies.

2.3 Constructs

The SV40-luciferase reporter, containing five copies of the GAL4-UAS, was obtained from J. Milbrandt [109]. pRL-TK was purchased from Promega. The GAL4-mammalian expression construct, GAL4-DBD (DNA-binding domain), has been described previously [104]. The Smyd2 and Smyd3 full-length EST clones were obtained from Invitrogen. The GAL4-Smyd2 mammalian expression

vector was constructed by PCR amplification (5’ ATG CGC GCC GAG GCC CGC; 3’ TCA GTG GCT CTC AAT CTC CTG) and restriction digestion (Not I; Xba I) followed by subcloning into the GAL4-DBD plasmid [104]. The Myc-Y240F and Myc-Y239F point mutations were generated within the SET domain of Myc-Smyd2 (primers: 5’ CGA GGT GTT CAC CAG CTT CAT CGA CCT GCT ATA TCC; 3’ GGA TAT AGC AGG TCG ATG AAG CTG GTG AAC ACC TCG) and Myc-Smyd3 (primers: 5’ GGA GCT CAC CAT CTG CTT CCT GGA CAT GCT GAT GAC C; 3’ GGT CAT CAG CAT GTC CAG GAA GCA GAT GGT GAG CTC C), respectively, using the GeneEditor *in vitro* Site-Directed Mutagenesis System (Promega) according to the instructions of the manufacturer. The FLAG-tagged HDAC1-F mammalian expression plasmid was a kind gift from S.L. Schreiber [110]. The FLAG-tagged MBD3-F and MTA2-F expression plasmids were supplied by R.M. Evans [111]. HELZ, MICAL and FKBP38 were kind gifts from the laboratory of our collaborator, Dr. Deepak Srivastava [106, 112, 113]. Frizzled 2 was provided by the laboratory of Dr. M. Sheng [114]. GST-Smyd3 was constructed by PCR amplification using the Smyd3-GAL4 template (primers: 5’ GGA TTC ATG GAG GCA CTG AAG GTG; 3’ GAA TTC TTA GGA GGC TCG TAT TTG GGC) and subcloning into pGEMT-EZ (Promega). The resulting construct was digested with BamHI and EcoRI, followed by subcloning of the insert into pGEX-6P-1 (Promega). The skNAC E34-GST construct was obtained by excision of E34 from the FLAG-E34 construct (previously described) [105] followed by subcloning of the fragment into pGEX-6P-1 (Promega). The RNAPII

CTD expression construct used has been previously described [115]. The sequences of all constructs were confirmed by DNA sequencing.

2.4 Transient transfections and luciferase assays

All transfections were performed using FuGENE6 reagent (Roche), according to the instructions of the manufacturer. For immunoprecipitation experiments, 293T cells were plated at a density of 2 X 10^6 cells per 100 mm plate, 24 hours prior to transfection. 8 µg of total DNA plus 24 µl of FuGENE6 reagent were used per 100 mm plate. Cells were harvested 48 hours after transfection. For luciferase reporter assays, 10T1/2 cells were seeded at a density of 2 X 10^5 cells per 6-well plate 24 hours prior to transfection. 3.5 µg of total DNA plus 10.5 µl of FuGENE6 reagent were used per well. Luciferase assays were performed using the Dual-Luciferase Reporter Assay System kit (Promega) according to the manufacturer's directions. Samples were analyzed using a Dynex microtiter luminometer.

2.5 Antibodies

The anti-Myc monoclonal (9E10), anti-HA monoclonal (H9658), and anti-FLAG monoclonal (M2) antibodies were purchased from Sigma-Aldrich. The anti-GAL4 mouse monoclonal (RK5C1) and the anti-Sin3A rabbit polyclonal (K-20) antibodies were purchased from Santa Cruz Biotechnology. Antibodies for

determining the specificity of HMT activity are detailed below. The anti-RNA Pol II antibody (clone 8WG16) was obtained from UpState (Charlottesville, VA). Peroxidase-conjugated whole IgG secondary antibodies were purchased from Jackson ImmunoResearch Laboratories.

2.6 Multiple tissue northern analysis

Northern blotting was performed using ULTRAhyb Hybridization Buffer (Ambion) according to the instructions of the manufacturer. The probes for full-length Smyd2 and Smyd3 were generated by restriction digest excision (Not I; Xba I) from their respective GAL4 mammalian expression constructs and the Strip-EZ DNA Probe Synthesis Kit (Ambion) as described in the manual. The probe for full length Smyd1 was generated by restriction digest excision (EcoRI) from the pBK-CMV-Smyd1B expression construct described previously [105]. The multiple tissue Northern blot was purchased from Ambion (FirstChoice Northern Blot Mouse Blot I). Blots were detected using a phosphoimager.

2.7 *In situ* hybridization

The DNA probe for full-length Smyd2 was generated by restriction digest excision (Not I; Xba I) from its above-described GAL4 mammalian expression construct. Probe synthesis, hybridization, and autoradiography were performed as described in Lu et al. [116] with slight modifications. Briefly, embryos were

obtained at day 13.5 post-coitus and fixed overnight in 4% paraformaldehyde. Hybridization of tissue sections with sense and antisense DNA probes was performed overnight at 55˚C, $7.5x10^5$ cpm per slide. Unhybridized probe was removed through stringent washing and slides were coated with K.5 nuclear emulsion (Ilford, UK) followed by exposure at 4˚C for 21 days. Once developed, slides were counterstained with hematoxylin and observed by bright and dark field optics.

2.8 Cellular localization by fluorescence imaging

293T cells were transfected with 1µg of plasmids, encoding myc tagged Smyd2, using Fugene 6 (Roche), according to the manufacturer's instructions. 48 hr post-transfection, cells were fixed (4% paraformaldehyde in PBS for 10 min), washed, and permeabilized (0.5% Triton X-100 in PBS for 15 min). Monoclonal mouse anti-myc antibody was incubated 45 min at room temperature. After washing, goat anti-mouse Alexa 488 (Molecular Probes) was diluted 1:400 in 2% BSA and incubated 15 min at room temperature in the dark. Nuclei were counterstained with DAPI. Slides were washed twice for 45 min and mounted with VectaStain. Cells were analyzed by sequential confocal laser scanning microscopy (Leica SP2 AOBS).

2.9 Cell proliferation assay

NIH3T3 cells were transfected with 6µg of Smyd1-Myc, Smyd2-Myc, Smyd3-Myc, or pcDNA3 alone. The effects of the over-expression of each protein on cell growth were observed over a 6-day period and evaluated by cell counting using trypan-blue exclusion.

2.10 *In vitro* histone methyltransferase assay

293T cells were transfected with plasmids expressing Myc-tagged wildtype Smyd2 (Smyd2-Myc), wildtype Smyd3 (Smyd3-Myc), mutant Smyd2 (Smyd2 (Y240F)-Myc), or mutant Smyd3 (Smyd3 (Y239F)-Myc) and the over-expressed, tagged proteins were purified by immunoprecipitation with an anti-Myc antibody. The histone methyltransferase assay was performed as described in Hammamoto et al. [106]. Briefly, the Smyd proteins were incubated with 1µg of mixed histones from calf thymus (Sigma) or recombinant H3 (Upstate). In addition, 2µCi S-adenosyl-L –[methyl-^{3}H] methionine (SAM; Amersham Biosciences) was included as a methyl donor. All reactions were carried out in 40µl HMT reaction buffer (10mM dithiothreitol, 100mM NaCl, 4mM MgCl2, and 50mM Tris-HCl at pH 8.8) at 30°C for 3 hours. An 18% SDS-PAGE gel was used to resolve the samples and fluorography was used visualize positive methylation. Substrate loading was visualized by Coomassie blue staining.

To determine the specificity of Smyd2 activity, immunoprecipitated Smyd proteins were incubated with recombinant H3 and 20μM unlabelled SAM (Sigma) in 40μl HMT reaction buffer at 30°C for 1 hour. Western blot analysis was conducted using antibodies against dimethylated H3K4, trimethylated H3K4, dimethyl H3K9, trimethyl H3K9, trimethyl H3K27, dimethyl H3K36, or dimethyl H3K79 (all from Upstate, Charlottesville, VA).

Proximity bead HMT assays were conducted as follows: 2μCi of the methyl-donor S-adenosyl-L–[methyl-^{3}H] methionine (SAM; Amersham Biosciences) were incubated with .1μg of Smyd3 and .1μg of a Histone H3 peptide, in which lysine 4 is mono-methylated (sequence of the peptide: acetyl-RT**K**QTARKSTGGKAPRK-biotin). The assay was carried out for three hours at 30°C in 20μl HMT reaction buffer. At the end of the incubation time, 100μl of binding buffer (1x PBS containing 1% NP-40 and 0.1% SDS) was added. The substrate was then precipitated using 10μl of Streptavidin PVT SPA Scintillation Beads (Amersham Biosciences; used as 50% slurry in binding buffer) for one hour at room temperature on a rocking platform, followed by five washes in binding buffer and scintillation counting.

2.11 Immunoprecipitations

Protein-A on Sepharose CL-4B was obtained from Sigma and 300mg were washed in 10ml low IPB buffer (150mM NaCl; 2mM EDTA; 0.5% NP-40; 25mM Tris, pH7.5;) containing 10mg BSA. The wash was carried out overnight

at 4°C while rotating. The protein-A beads were subsequently washed 2 times in 10ml low IPB buffer, before being resuspended in 3ml low IPB. Protein G-PLUS Agarose was obtained from Santa Cruz Biotechnology. 293T cells were transiently transfected and later harvested, 48 hours after transfection. Cells were collected and lysed in RIPA buffer (150mM NaCl, 1% NP-40, 0.5% DOC, 50mM Tris pH 8, 0.1% SDS) containing protease inhibitors for 30 minutes on ice, followed by brief sonication. Protease inhibitor cocktail tablets (mini-complete; Roche Molecular Biochemicals, Indianapolis, IN) were included for all incubations and washes. Cells were centrifuged (16,000g) for 10 minutes at 4° C and supernatants were incubated with primary antibody (0.5 – 2ug/ml) on ice for one hour. Lysates were subsequently centrifuged at 4°C for 10 minutes and the supernatants were incubated with 25µl protein A-Sepharose/protein G PLUS-agarose (25µl) at 4°C with rotation for 1 hour. Resulting immune complexes were washed 6 times with 1ml low IPB and immunoprecipitated proteins were resuspended in 40µl of 2X SDS loading buffer (20% glycerol, 250 mM Tris, pH 6.8, 2%SDS, 200 mM β-mercaptoethanol, bromophenol blue to color), boiled for 5 minutes, and resolved by 8-15% SDS-PAGE.

2.12 Western blot analysis

After separation by SDS-PAGE, proteins were transferred to a nitrocellulose membrane (Protran BA, Schleicher and Schuell, NH) using electrophoretic transfer [117]. Membranes were blocked using 5% nonfat milk

(10g nonfat milk, 150 mM NaCl, 10 mM Tris pH 8, 0.05% Tween-20) overnight at 4°C. Membranes were later incubated with 1° antibody for 1 hour at room temperature followed by washing in TBS-T (150mM NaCl, 20mM Tris pH 8, 0.1% Tween-20) four times for 15 minutes. Membranes were then incubated with 2° antibodies for 1 hour at room temperature with and subsequently washed with TBS-T four times for 15 minutes. Blots were exposed and developed using the ECL blot detection reagent (Amersham Pharmacia Biotech) according to the instructions of the manufacturer.

2.13 Cloning of the Smyd2 conditional targeting construct

To construct the Smyd2 conditional targeting construct, two genomic fragments were first subcloned from the C57BL/6 murine Bac clone-RPC124288J3. A 2.2kb KpnI fragment containing exon 1 and a KpnI fragment containing 5.2kb of intronic sequence between exons 1 and 2 was subcloned into pBluescript (Stratagene). Fragment 1 (5.2kb) was excised with KpnI, blunt ended, and ligated into the unique blunt ended SalI site of pDELBOY [118]. The resulting clones were screened for correct orientation and for the regeneration of the SalI site. Fragment 2 (2.2kb) was excised with KpnI and ligated into the unique KpnI site of pDELBOY containing fragment 1. This was subsequently screened for correct orientation. Fragment 3, containing 0.6kb upstream of exon 1, was generated using Platinum Pfx DNA Polymerase (Invitrogen), C57BL/6 genomic DNA as template, and the following primer pair:

5’GTCGACATTGAGCTAATGTGCTTA-3’;

5’-CTCGAGGTAACACTCAACCTCTGC-3’.

The resulting PCR product was treated with TAQ Polymerase, ligated into pGEMTEASY (Promega), and excised with SalI and XhoI. This product was ligated into the unique XhoI site of pDELBOY containing fragments 1 and 2 and subsequently screened for correct orientation. The completed targeting construct was linearized at the short arm of homology using XhoI. C57BL/6 ES cells were then transfected and selected with G418 and gancyclovir. Targeted ES cell colonies were screened by Southern hybridization analysis using probes specific for the genomic sequence external to the arms of homology. The 5’ Southern used a 0.8kb PCR fragment using the following primer pair:

5’-GGCTGGAGTTAGAGGTGGTTATGA-3’;

5’-ACAGCTCTGGGCTCGGAAATAAAG-3’.

The 3’ Southern used a 0.9 kb PCR fragment using the following primer pair:

5’-AACTCCATGTGGTGGAATTCTGTGGT-3’;

5’-GCAGCCTGAAAGAATCCCTTAGACT-3’.

2.14 Acid histone extraction

Cells ($4x10^{7)}$ were washed twice with ice-cold PBS and resuspended in Triton extraction buffer (TEB: PBS containing 0.5% Triton X (v/v), 2mM

phenylmethylsulfonyl fluoride, 0.2% NaN3 (v/v)). Cells were lysed (H-Lysis solution**:** 0.25M sucrose, 3 mM CaCl2, 1 mM Tris pH8.0, 0.5% NP-40) on ice for 10 minutes and subsequently centrifuged at 2000rpm for 10 minutes at 4°C. The pellet was washed in half the volume of TEB and centrifuged as before. The product was washed in wash solution (300 mM NaCl, 5mM MgCl2, 5mM DTT, 0.5% NP-40) and centrifuged as before. Acid extraction was conducted at 4° overnight in extraction buffer (0.5M HCl, 10% glycerol, 0.1M 2-mercaptoethylamine-HCl). Samples were centrifuged as before and protein concentration was determined by Bradford assay.

2.15 Affinity selection mass spectrometry

Affinity selection–mass spectrometry (ASMS) was employed for screening pools of small molecules for their ability to interact with Smyd3. Precise methods are detailed in [119]. Briefly, Smyd3 was incubated with known libraries of potentially interacting small molecules (incubation buffer included 50 mM Tris, pH 7.5 with 5% DMSO). The product was injected into a mobile phase of 50 mM NaH_2PO_4, pH 7.5, and complexes were separated from unbound components using size exclusion chromatography. Small molecules were then dissociated from Smyd3 with 5% CH_3CN/95% H_2O in 0.2% formic acid and identified based on their known library of origin and mass spectrometry.

2.16 Hsp90Plus™ genotype

F-hsdS gal omp T (λ cI857 ind-1 nin-5 Sam-7 lacUV5-T7 gene 1) Lon- . *E. coli* B strain with a λ prophage carrying the T7 RNA polymerase gene under the lacUV5 promoter and a pACYC177-derived chloramphenicol-resistant plasmid expressing full-length human Hsp90α or Hsp90β. These strains were obtained from Expression Technologies, Inc. and handled according to the instructions of the manufacturer.

2.17 Expression and purification of soluble GST-fusion proteins

GST-Smyd2, GST-Smyd3, and GST-RNAPII CTD were transformed into Plus90α™, Plus90β™ (Expression Technologies, Inc., San Diego, CA), or standard BL-21 competent *E. coli* cells and selected according to the manufacturer's instructions (www.exptec.com). Transformants were inoculated into 10ml of LB broth, containing 150μg/ml ampicillin and cultures were left to shake at 37˚C overnight. Overnight cultures were used to inoculate 250ml of LB broth, containing 150μg/ml ampicillin and cultures were incubated on a shaker at 37˚C until OD_{600} reached 0.5. At that time, 100mM IPTG was added to a final concentration of 0.1 mM and cultures were grown for an additional 5 hours at 22˚C. Cells were then pelleted at 5000 X g for 10 minutes, and 2.5g of wet cell paste was isolated from each culture pellet. The isolated cell paste was resuspended and lysed at room temperature in 12.5ml of BugBuster HT

(Novagen) containing a protease inhibitor cocktail (Roche), according to the manufacturer's instructions. Lysis proceeded for 20 min. on a shaking platform followed by centrifugation at 10,000 X g for 10 min. at 4˚C. The supernatant was transferred to a clean flask containing 25ml binding buffer (20mM Hepes [pH 7.6], 100mM KCl, 20% glycerol, 1mM DTT, 1mM EDTA, and 1X protease inhibitor cocktail [Roche]) and 3.5ml 10% triton X-100. To this, 1ml of a 50% slurry of glutathione agarose beads (Sigma-Aldrich), equilibrated with binding buffer, was added to the mixture and incubated on a rocking platform for 8 hours at 4˚C. Beads were subsequently centrifuged at 2,000 X g and washed 6 times in 20ml binding buffer that contained no protease inhibitors. Beads were then washed 4 times in PreScission (Amersham Bioscience) cleavage buffer (prepared as described in the manufacturer's protocol) and GST- cleavage was accomplished using PreScission Protease (Amersham Bioscience) according to the manufacturer's protocol. Purity of the products was verified using silver staining of SDS-PAGE gels.

2.18 Expression and purification of insoluble GST-fusion proteins

Expression of insoluble GST-fusion proteins was performed as described above. For purification of insoluble GST-fusion proteins, the pellets obtained from the post-lysis centrifugation were resuspended in 12.5ml of BugBuster (Novagen). Lysozyme was added to a final concentration of 200µg/ml and the inclusion bodies were isolated according to the BugBuster protein extraction protocol

(Novagen). GST-fusion proteins were treated using the Novagen Protein Refolding Kit, according to the directions of the manufacturer. Refolded fusion proteins were purified on glutathione beads and the GST-moiety was subsequently cleaved, as described above.

2.19 Smyd polyclonal antibody production

Smyd1 polyclonal antibodies were generated by immunizing New Zealand White rabbits four times each with 50µg Smyd1B-6XHis protein produced in insect cells and purified on a nickel column. Rabbit serum was collected before immunization as a negative control. The specificity of the Smyd1 antibody was tested by comparing its reactivity on western blots arrayed with lysates from 293T cells over-expressing Smyd1B fusion proteins versus lysates of 293T cells over-expressing Smyd2 and Smyd3 fusion proteins. At a dilution of 1:1000, the Smyd1 polyclonal antiserum was highly reactive against Smyd1 fusion proteins but completely inert toward Smyd2 and Smyd3 fusion proteins.

Smyd2 polyclonal antibodies were generated by immunizing New Zealand White rabbits four times each with 50µg Smyd2 protein produced in *E. coli* as GST-fusions and purified on a GST-column as detailed in 2.17. The GST-moiety was cleaved as part of the purification process, as described in 2.17, prior to the injections. Rabbit serum was collected before immunization as a negative control. The specificity of Smyd2 antibody will be tested as described above.

Smyd3 polyclonal antibodies were prepared as detailed above for Smyd1. The specificity of the Smyd3 polyclonal antibodies is demonstrated in chapter 4.

3. IDENTIFICATION AND CHARACTERIZATION OF SMYD2

3.1 Abstract

Disrupting the balance of histone lysine methylation alters the expression of genes involved in tumorigenesis including proto-oncogenes and cell cycle regulators. Methylation of lysine residues is commonly catalyzed by a family of proteins that contain the SET domain. Here, I report the identification and characterization of the SET domain-containing protein, Smyd2.

Smyd2 mRNA is most highly expressed in heart and brain tissue, as demonstrated by northern analysis and *in situ* hybridization. Over-expressed Smyd2 localizes to the cytoplasm and the nucleus in 293T cells. Although accumulating evidence suggests that methylation of histone 3, lysine 36 (H3K36) is associated with actively transcribed genes, I show that the SET domain of Smyd2 mediates H3K36 dimethylation and that Smyd2 represses transcription from an SV40-luciferase reporter. Smyd2 associates specifically with the Sin3A histone deacetylase complex, which was recently linked to H3K36 methylation within the coding regions of active genes in yeast. Finally, I report that exogenous expression of Smyd2 suppresses cell proliferation.

I propose that Sin3A-mediated deacetylation within the coding regions of active genes is directly linked to the histone methyltransferase activity of Smyd2. Moreover, Smyd2 appears to restrain cell proliferation, likely through direct modulation of chromatin structure.

3.2 Background

Cell proliferation and differentiation are coordinated by synchronized patterns of gene expression. The regulation of these patterns is achieved, in part, through epigenetic mechanisms that affect the nature of DNA packaging into chromatin [98]. Specifically, post-translational covalent modifications to histone tails impact the structural dynamics of the nucleosome, thereby affecting DNA accessibility to transcriptional complexes [60, 120, 121]. Common modifications to histones include methylation, acetylation, phosphorylation, and ubiquitination [85]. Accumulating evidence suggests that transcriptional regulators facilitate these alterations, resulting in altered local gene transcription [85]. Thus, the structural interpretation of histone modifications may be responsible for the establishment and maintenance of discrete programs of gene expression that ultimately correspond with distinct biological outcomes. Of equal importance, aberrations in global levels of histone methylation and acetylation are connected to the biology of cancerous lesions and their clinical outcome [122]. Although a number of histone lysine methyltransferases (HKMTs) are disrupted in a variety of cancer types [106, 123], how histone methylation mechanistically contributes to the oncogenic state is poorly understood.

The majority of HKMTs catalyze methyl transfer via the SET domain, a module encoded within many proteins that regulate diverse processes, including those critical for development and proper progression of the cell cycle [27, 60,

84, 124]. Histone lysine methylation on specific residues typically correlates with distinct states of gene expression [85]. Histone 3 (H3) contains most of the known targeted lysines of histone methyltransferases and thereby serves as a conduit of such epigenetic regulation. In general, lysine methylation on H3K9, H3K27, or H4K20 corresponds with gene silencing, whereas methylation of H3K4, H3K36, or H3K79 is associated with actively transcribed genes [85].

Methylation of H3K36 which has been associated with actively transcribed genes [124, 125], appears to correspond primarily within coding regions. H3K36 methylation (H3K36me) by Set2 in yeast was recently observed to recruit an Rpd3-mediated histone deacetylase complex through direct recognition of H3K36me by the chromodomain of the Eaf3 subunit of Rpd3 [126-128]. Rpd3 is a histone deacetylase (HDAC) that has well-established functions as a transcriptional repressor [126]. It associates with several co-repressor complexes, including one that contains Pho23, Sds3, Sap30, Ume1, Cti6/Rxt1, and Sin3 [126]. However, recent evidence suggests that HDACs may also play a role during active transcription. As such, methylation of H3K36 is directly linked to histone deacetylation, via Rpd3-Sin3, that in turn functions to maintain chromatin structure during active transcription [126-128]. These findings reveal a new level of complexity with respect to histone modifications, and demonstrate our need to better understand the enzymes that catalyze these modifications.

Here, Smyd2 is identified as a member of a subfamily of SET domain containing proteins. As described in section 1.7, the Smyd family of proteins is defined by a SET domain that is split into two segments by an MYND domain,

followed by a cysteine-rich post SET domain [104]. While Smyd1 and Smyd3 have been previously characterized [104, 106], nothing was known about the biochemical or functional properties of Smyd2, prior to these studies. The findings reported here demonstrate that Smyd2 contains SET-domain dependent H3K36 HKMT activity. Smyd2 specifically associates with the Sin3A histone deacetylase complex, suggesting a link between two independent chromatin modification activities. Moreover, over-expression of Smyd2 in fibroblasts is shown to significantly suppress their growth. It is therefore proposed that Smyd2-mediated chromatin modification regulates specific gene expression that has important implications for normal and neoplastic cell proliferation.

3.3 Results

3.31 Structural characteristics and expression of Smyd2

Although there are over 50 SET domain-containing proteins encoded in the human genome, only a fraction have been shown to methylate histones. Of all the SET proteins, five cluster into a sub-family, Smyd, that contains a **S**ET domain that is split into two segments by a **MY**N**D** domain/zinc-finger motif, implicated previously in protein-protein interactions (Fig. 3.1A & C). Members of this family are direct regulators of cancer (Smyd3) and essential developmental processes (Smyd1) [104, 106]. Thus, it is important to discern the biochemical and biological properties of Smyd2, given its high degree of homology to Smyd1 and Smyd3. Data from Expressed Sequence Tags suggest that Smyd2 is

expressed in a wide range of normal, tumor, and diseased tissues. To determine the tissues of highest gene expression, northern blotting was performed with a multiple tissue blot using probes specific to Smyd1, Smyd2, or Smyd3. The northern analyses (Fig. 3.1B) demonstrate that in contrast to Smyd1, Smyd2 and Smyd3 transcripts are expressed more broadly in a wide variety of tissues. Smyd1 is expressed only in T lymphocytes, cardiac muscle, and skeletal muscle, as previously reported [129]. Smyd3 expression is highest in skeletal muscle [106] and thymus (Fig. 3.1B), although its transcripts are also highly detected in the brain, kidney, and ovary (Fig. 3.1B). Tissues containing the highest levels of Smyd2 mRNA transcripts include heart, brain, liver, kidney, thymus, and ovary (Fig. 3.1B.) Additionally, both Smyd2 and Smyd3 transcripts are detectable in embryonic mRNA, suggesting that as with Smyd1, Smyd2 and Smyd3 may be involved in development (Fig. 3.1B). To determine which embryonic tissues manifest highest levels of Smyd2 transcripts, whole-mount *in situ* hybridization was performed using murine embryos at day 13.5 with a probe specific to Smyd2. At this mid-gestation stage, Smyd2 transcripts are localized to the heart and the hypothalamus of the brain (Fig. 3.1E).

Immunohistochemical staining of Smyd1 and Smyd3 indicated that both proteins localize within the cytoplasm and nucleus of C2C12 [105] and Huh7 cells [106], respectively. To determine the subcellular localization of Smyd2, immunohistochemical staining was performed using a myc-tagged Smyd2 fusion protein. Similar to Smyd1 and Smyd3, Smyd2 localizes within both the cytoplasm and the nucleus (Fig. 3.1D).

3.32 Smyd2 is a SET-dependent HKMT

Smyd2 contains the catalytic core residues of the SET domain shown to be critical for the histone methyltransferase activity of Smyd1 and Smyd3 (Fig. 3.1C) [106, 130]. This suggests that Smyd2 may also possess HKMT activity. Histone methylation was tested after incubation of wild-type Smyd2-Myc (or Smyd3-Myc as a positive control) with S-adenosyl-L –[methyl-^{3}H] methionine (SAM) and mixed histones from calf thymus as a substrate. A 17kD band corresponding to ^{3}H-labelled H3 was seen with both Smyd3 and Smyd2 in the fluorogram (Fig. 3.2A, lanes 1 and 3), indicating that Smyd2 has intrinsic HKMT activity. A tyrosine in the C-terminal region of the core SET domain is conserved among catalytically active SET domain proteins (Fig. 3.1C). Therefore, to test if the SET domain is required for the HKMT activity observed for Smyd2, point mutations were made in this residue of Smyd3-Myc (Y239F) and Smyd2-Myc (Y240F). Neither Smyd3 (Y239F)-Myc nor Smyd2 (Y240F)-Myc displayed HKMT activity (Fig. 3.2A, lanes 2 and 4).

Smyd2 HKMT activity was next tested on a recombinant histone H3 substrate. Wild-type Smyd2-Myc methylated H3 (Fig. 3.2C, lane 1) but Smyd2 (Y240F)-Myc showed no HKMT activity (Fig. 3.2C, lane 2). To test the activity of untagged Smyd2 protein, Smyd2 was expressed as a GST fusion in E. coli and the GST moiety was subsequently cleaved. Methylation of recombinant histone octamers was tested after incubation with recombinant Smyd2. Consistently,

recombinant Smyd2 was observed to methylate histone H3 (Fig. 3.2B, lane 1). Therefore, it is concluded that the Smyd2 has SET domain-dependent, intrinsic HKMT activity.

3.33 Smyd2 dimethylates H3K36

Given that Smyd3 has been shown to have specificity for H3K4 [106], I tested whether Smyd2 has similar specificity. Histone methyltransferase assays were performed using recombinant H3, and specificity was determined by western blotting using antibodies against various methyl-lysine residues. The antibodies used in these assays include anti-dimethyl H3K4, anti-trimethyl H3K4, anti-dimethyl H3K9, anti-trimethyl H3K9, anti-trimethyl H3K27, anti-dimethyl H3K36, and anti-dimethyl H3K79. A 17KD band corresponding to H3 was observed with Smyd3, but not with Smyd2, when reactions were probed with anti-dimethyl H3K4 (Fig. 3.2D, lanes 4 and 1, respectively) or anti-trimethyl H3K4. This indicated that Smyd2 has a different target specificity than Smyd1 or Smyd3. Instead, the HKMT activity of Smyd2 was specific for H3K36, as determined by western blotting with anti-dimethyl H3K36 antibodies (Fig. 3.2E, lanes 1 and 4, respectively). No additional residues appeared to be targeted by Smyd2 using the other antibodies listed above. Therefore, it is concluded that Smyd2 dimethylates H3K36.

3.34 Smyd2 associates with HDAC1 and the Sin3 repression complex

Smyd3 induces transcriptional activation by binding to specific promoter sequences [106]. In contrast, when fused to GAL4, Smyd1 is known to repress transcription in an HDAC dependent manner [106]. Given that Smyd2 has activity towards H3K36, a mark associated with active transcription, the transcriptional regulatory activity of Smyd2 was tested. A GAL4-fusion protein was generated using Smyd2 and transient luciferase assays were performed in 10T1/2 cells. Unexpectedly, Smyd2-GAL4 inhibited transcription from an SV40 promoter that contained GAL4 binding sites (Fig. 3.3B), suggesting it may function similarly to Smyd1.

Although methylation of H3K36 is associated with actively transcribed genes, three recent reports have demonstrated that in yeast, methylation of H3K36 by Set2 recruits an Rpd3-Sin3 histone deacetylase complex [126, 128]. The finding that Smyd2 possesses H3K36 methylation activity and functions to repress transcription in the above assay prompted us to investigate whether Smyd2 interacts with the human homologs of the Rpd3-Sin3 complexes. In transient transfection experiments in 293T cells, the human homolog of Rpd3, HDAC1, interacted specifically with Smyd2-GAL4 upon immunoprecipitation with anti-GAL4 antibodies (Fig. 3.3C). Consistently, when cell extracts from 293T cells over-expressing Smyd2-GAL4 were immunoprecipitated with anti-GAL4 antibody, the immune complexes contained endogenous Sin3A (Fig. 3.3D). In contrast, Smyd2-GAL4 failed to coimmunoprecipitate FLAG-tagged MBD3 and MTA2, components of the HDAC1-containing NuRD complex (Fig. 3.3D). Thus,

it is concluded that Smyd2 preferentially interacts with distinct HDAC1-containing complexes, namely Sin3A.

3.35 Smyd2 suppresses cell proliferation

The role of Smyd3 in transcriptional regulation as a histone methyltransferase has been linked to its ability to augment cellular proliferation [106]. To investigate the effects that Smyd2 may have on cell proliferation, NIH3T3 cells were transfected with either Smyd2-Myc or Smyd3-Myc. Relative to a mock control and consistent with previous findings [106], over-expression of Smyd3 markedly increased cell growth (Fig. 3.4). Conversely, the transfection of 3T3 cells with Smyd2 led to a decrease in their proliferation (Fig. 3.4), indicating a potential role for Smyd2 in the maintenance of cell-cycle progression.

3.36 Status of Smyd2 conditional knockout

Targeted deletion of Smyd2 will be accomplished by crossing tissue-specific or ubiquitously expressing Cre into Smyd2 homozygous floxed mice. The targeting vector (Fig. 3.5A) contains a short (0.6kb) and a long (7.4 kb) arm of homology, a neomycin resistance cassette (neo) for positive selection, and a thymidine kinase cassette for negative selection. Two site-specific recombination sites are employed *in vivo*. (1) Flp recombinase is used to delete the neo marker from the mouse germline, and (2) Cre recombinase is used to conditionally

delete Smyd2 in selected tissues. Two loxP sites flank the region to be deleted. This region includes exon 1 of Smyd2.

Details relevant to the creation of the Smyd2 conditional targeting construct are provided in the Materials & Methods (chapter 2.13). Briefly, to create the Smyd2 conditional targeting construct, two genomic fragments were first subcloned from a C57BL/6 murine Bac clone. These included a 2.2kb fragment containing exon 1 and a fragment containing 5.2kb of intronic sequence between exons 1 and 2. A third fragment, containing 0.6kb upstream of exon 1, was generated using C57BL/6 genomic DNA as template (primers provided in chapter 2.13). Each fragment was consecutively inserted into unique sites of a destination targeting construct (pDELBOY [118]) and the completed targeting construct was linearized at the short arm of homology. C57BL/6 ES cells were then transfected and selected with G418 and gancyclovir.

Successfully targeted ES cells were identified by Southern analyses. These assays were performed across the long arm of homology (Fig. 3.5B) and the short arm of homology (Fig. 3.5C) of clones that had experienced homologous recombination at the Smyd2 locus. A size difference allowed the separation of targeted allele and wild type allele. These clones were then injected into C57Bl/6J-tyr©-2J (an albino strain) blastocysts, which were implanted into the uteri of psuedopregant recipients and ultimately chimeras were born. The chimeras were mated to albino C57Bl/6 females and resulting progeny with a black coat color were genotyped. Mice that were shown to be

heterozygous for the targeted allele were mated to Flip recombinase-expressing transgenic mice to remove the neo cassette.

Presently, we have 5 mice in which the neo cassette has been removed (floxed). They are currently being crossed with wild-type C57Bl/6 mice to remove the flp transgene. In a tandem effort to analyze a Smyd2 hypomorph, in which the presence of a neo cassette disrupts the promoter region of Smyd2, we have bred one mouse to homozygosity for the presence of neo. This male mouse exhibits no marked phenotype, to date. It will be crossed with females which are heterozygous at this allele in a series of timed matings to obtain mouse embryonic fibroblasts (MEFs) which are homozygous for the presence of neo. Although the our ultimate goal is to produce floxed/floxed homozygous mice from which Smyd2 can be conditionally removed, we hope that obtaining hypomorphic MEFs in the meantime will provide us with an intermediate cell line that will facilitate early *in vivo* analyses on the effects of disturbing the expression of Smyd2.

3.4 Discussion

Great strides have been made in the interpretation of covalent histone modifications regarding their role in transcriptional regulation. Histone lysine methylation has been found to affect the structure of chromatin thereby establishing complex patterns of gene expression [131]. In some cases, these patterns are clearly defined. For example, H3K4 methylation is most often

associated with the establishment of euchromatin and the consequent activation of local gene expression [121]. Reciprocally, methylation at H3K9 is commonly involved with the formation of heterochromatin and the ensuing silencing of nearby gene transcription [120, 121].

Initial data on the yeast HKMT, Set2, indicated that it functions in transcriptional repression by methylating H3K36 [132]. However, the HKMT activity of Set2 was later linked to the elongation phase of RNA polymerase II (RNAPII) [109, 133]. Likewise, in a more contemporary study, an analysis of the distribution of H3K36 methylation in metazoans correlated this modification with actively transcribed genes [124]. Most recently, methylation of H3K36 by Set2 has been associated with the recruitment of a histone deacetylase complex, Rpd3 [126]. The overall role and implications of histone deacetylation within the coding regions of active genes is still unclear.

In mammalian epigenetics, NSD1 was one of the first HKMTs reported to act on H3K36 [134]. Whether NSD1 functions in the activation or repression of transcription has yet to be determined. A recent investigation reported that the human HYPB protein methylates H3K36 and that this enzymatic activity is required for the role of HYPB as a transcriptional activator [135].

The present findings introduce Smyd2 as a H3K36-specific HKMT that acts as a transcriptional repressor. Clearly, there are other transcriptional regulatory mechanisms at work in conjunction with the methylation of H3K36. It seems that the more we learn about where histone marks are localized and what

proteins facilitate the process, the less we are certain about how such localization ultimately contributes to gene regulation. Although this complicates our ability to apply a broad interpretation of histone modifications, it provides a clear direction for the pursuit of a deeper fold in the histone code.

3.41 Smyd2 regulatory functions

Transcriptional assays demonstrated that Smyd2 can repress transcription from a luciferase reporter gene (Fig. 3B). A recent study in yeast demonstrated that methylation of H3K36 recruits a histone deacetylase complex, Rpd3 [126]. Concurrently, another group concluded that H3K36 methylation-induced recruitment of an Rpd3 complex resulted in the reversal of lysine acetylation related to the elongation phase of RNAPII, suggesting that it functioned to stem intragenic transcription initiation [128]. This is reminiscent of the mechanism by which the FACT complex functions. That is, as the elongation complex traverses a coding region, FACT facilitates both destabilization of the chromatin structure, to impart efficient and processive elongation, as well as reorganization of the chromatin to prevent intragenic initiation of transcription [136]. Whereas H3K36 methylation recruits the Rpd3 complex, it has been suggested that FACT recruitment may occur through its association with CHD1, which recognizes trimethylated H3K4 [137]. As the Rpd3 complex is known to contain Sin3 [126], it was particularly informative to find that Smyd2 also associates with Sin3. It will

be of further interest to determine whether *in vivo* recruitment of Sin3 requires H3K36 methylation, the presence of Smyd2, or both.

Over-expression of Smyd2 in NIH3T3 cells significantly reduces cell proliferation. In a previous study, cell proliferation assays demonstrated that Smyd3 augmented cell growth when introduced into NIH3T3 cells [106]. It is well established that cell proliferation and differentiation are coordinated by synchronized patterns of gene transcription. In the case of Smyd3, enhancement of cell growth has been shown to be dependent upon the HKMT activity of the Smyd3 protein [106]. It will be informative to determine whether the suppressive effect of Smyd2 on cell growth requires its function as an H3K36-specific HKMT. Such a determination, in tandem with identification of the putative gene target specificity of Smyd2, will provide a broader mechanistic model of how the Smyd family may function.

Histone lysine methylation is more stable than other known post-translational modifications, persisting as long as several rounds of cell division [131, 138, 139]. This makes lysine methylation potentially valuable in diverse, long-lasting signaling networks, not only in the nucleus for histone and non-histone proteins, such as p53 and TAF10, but conceivably in the cytoplasm as well. The findings that Smyd1 and Smyd3 can localize in the cytoplasm [105, 106] along with my observation that Smyd2 is also capable of cytosolic localization, lends credence to this idea. This argument is further strengthened by the finding that Smyd1 moves from the nucleus to the cytoplasm during myogenic differentiation [105]. Another SET domain-containing HKMT, Ezh2 and

its partners Eed and Suz12, reside primarily in the cytoplasm of various mouse and human cells [140, 141]. Within the nucleus, the Ezh2 complex catalyzes H3K27 methylation, whereas the cytosolic Ezh2 binds Vav1, a controller of Rho family GTPases, and Ezh2 is important for signaling events previously attributed to Vav1 [140-142]. There is no evidence that Ezh2 methylates Vav1, so the significance of lysine methylation in the cytoplasm remains unclear. Therefore, an important next-step in the characterization of Smyd2 will be to test the role of Smyd2-mediated lysine methylation in the formation of stable and potentially heritable cytosolic signaling complexes with Smyd2 interaction partners and to track these complexes, once formed, within resting and dividing cells.

3.42 Implications for the Smyd Family

The Smyd HKMTs are set apart from other such chromatin modifying enzymes by the split nature of their SET domains. The SET domain of each Smyd protein is divided by a MYND domain (Figure 3.1A & C), a zinc-finger motif that mediates protein-protein interactions. This domain is found in several transcriptional regulators shown to mediate distinct biological functions [107, 108]. For example, the MYND domain of Smyd1 is essential for its interaction with the muscle-specific transcription factor, skNAC [105]. Additionally, ETO, a common target of chromosomal translocations in acute myeloid leukemia, directs transcriptional repression through an intact MYND domain [107]. Thus, the importance of the MYND domain in gene regulation has been well established

and it may provide some insight into other mechanisms at work through Smyd2 that affect the overall outcome of its activity in transcriptional regulation. The complete function of Smyd2 *in vivo* is likely dependent upon other proteins and complexes, in addition to HDAC1 and Sin3A, with which it associates.

Northern blot analyses revealed that Smyd2 and Smyd3 are expressed in a wide variety of tissues (Fig. 1B) whereas Smyd1 is more restricted in its tissue distribution [129]. Studies of ours and others on Smyds1, 2, and 3 suggest that Smyd family members function through a common mechanism, specifically, lysine methylation. It is reasonable to assume that individual Smyd proteins associate with different transcription factors and other effector proteins that ultimately dictate specific gene regulation. The significant expression of Smyd2 in the in embryonic heart (Fig. 3.1B, E) suggests that as with Smyd1, Smyd2 may regulate cardiac development. The identification of the biological functions of Smyd family proteins will undoubtedly reveal new insights into the relationships between chromatin modifications and the development and differentiation of specific tissues.

3.5 Conclusion

I conclude that Smyd2 dimethylates H3K36 and that HDAC1-mediated deacetylation of the coding regions of active genes is potentially linked to the histone methyltransferase activity of Smyd2. I further propose that this role of Smyd2 in the regulation of gene expression ultimately restrains cell proliferation. As it is clear from this study, future research on Smyd proteins, with strong emphasis on the unique organismal context, will shed light onto the biological functions of Smyd family proteins, revealing new and fascinating insights into the relationships between chromatin modifications and the development and differentiation of tissues and organisms.

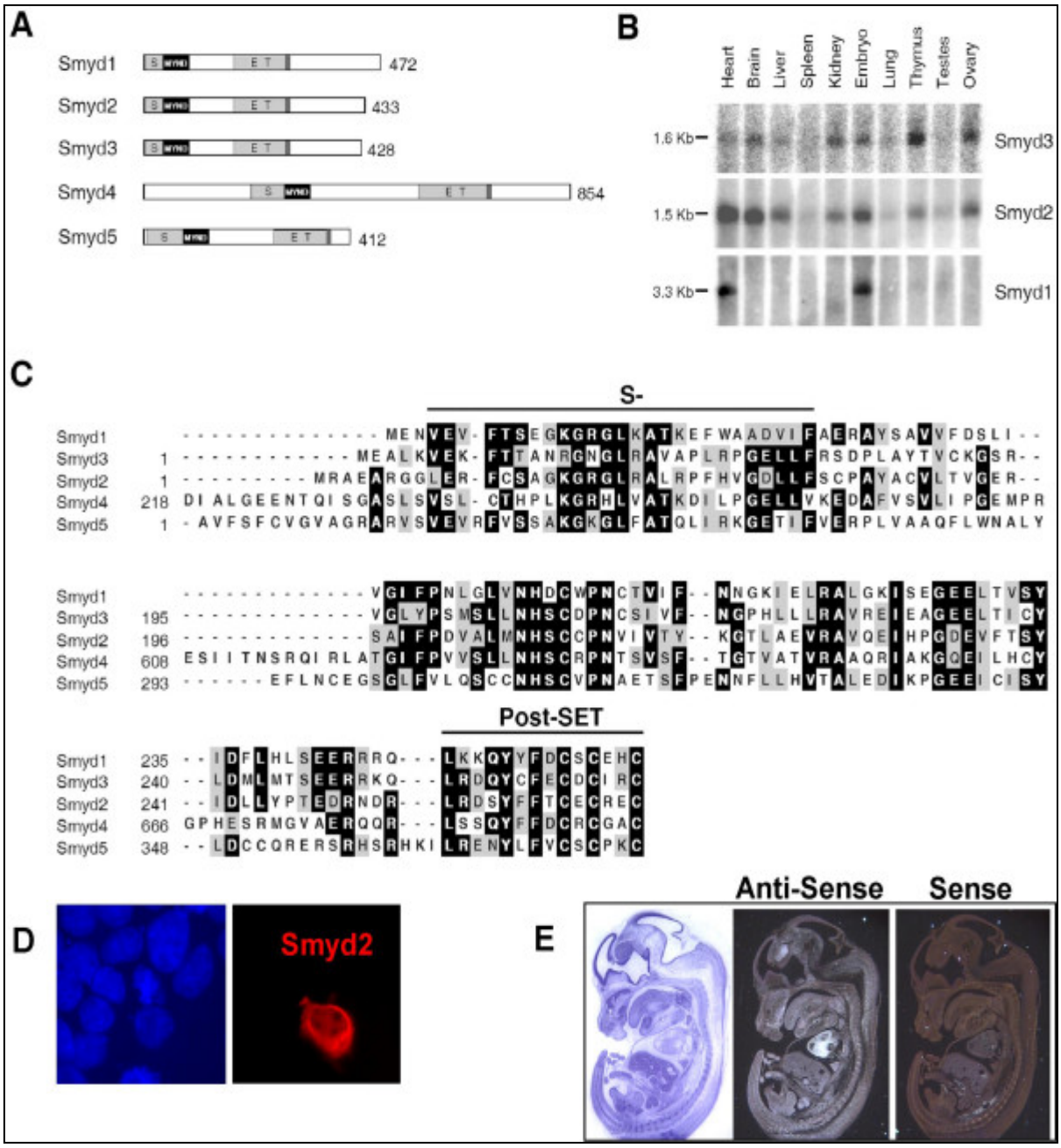

Figure 3.1 Alignment of the mammalian Smyd family proteins and Smyd2 localization. (A) Schematic representation of the five mammalian Smyd proteins. The split SET domain is shown in light gray; the MYND domain is represented in black and the cysteine-rich post-SET domain is displayed in dark gray. Positions of the amino acids are indicated. (B) Expression of Smyd1, Smyd2, and Smyd3 transcripts in tissues. Top panel: Smyd3 mRNA is most highly expressed in the thymus. Middle panel: Smyd2 mRNA is most highly expressed in the heart and

brain. Bottom panel: Smyd1 expression is restricted to the heart and skeletal muscle [104]. Transcripts for Smyd1, Smyd2, and Smyd3 are expressed in the embryo. (C) ClustalW alignment of the amino-terminal SET residues, the MYND domain, and the core SET residues followed by the post-SET domain present in Smyd1, Smyd2, Smyd3, Smyd4, and Smyd5. (D) Smyd2 is localized to the cytoplasm and the nucleus. Exponentially growing 293T cells were transfected with 1 μg of plasmids, encoding myc tagged Smyd2. 48 hr post-transfection, cells were fixed, washed, permeabilized and exposed to monoclonal mouse anti-myc antibody. Nuclei were counterstained with DAPI. Right panel: Smyd2 (red) localizes to both the nucleus and the cytoplasm of 293T cells. Left panel: Nuclei were counterstained with DAPI (blue). The experiments were repeated in triplicate with identical results. (E) Smyd2 mRNA is localized in the heart and hypothalamus of the brain at E13.5. Whole-mount *in situ* hybridization of Smyd2 transcripts in embryos at day 13.5 post coitus were prepared by exposition of sense (right panel) and antisense (middle panel) DNA probes, specific to Smyd2 to the sections. Whereas hybridization with sense probe resulted in no signal (right panel), thus, serving as control, Smyd2 mRNA is easily detected in the heart and the hypothalamus of the brain in embryos at day 13.5 post coitus (middle panel). (Adapted from Brown, et al. [100])

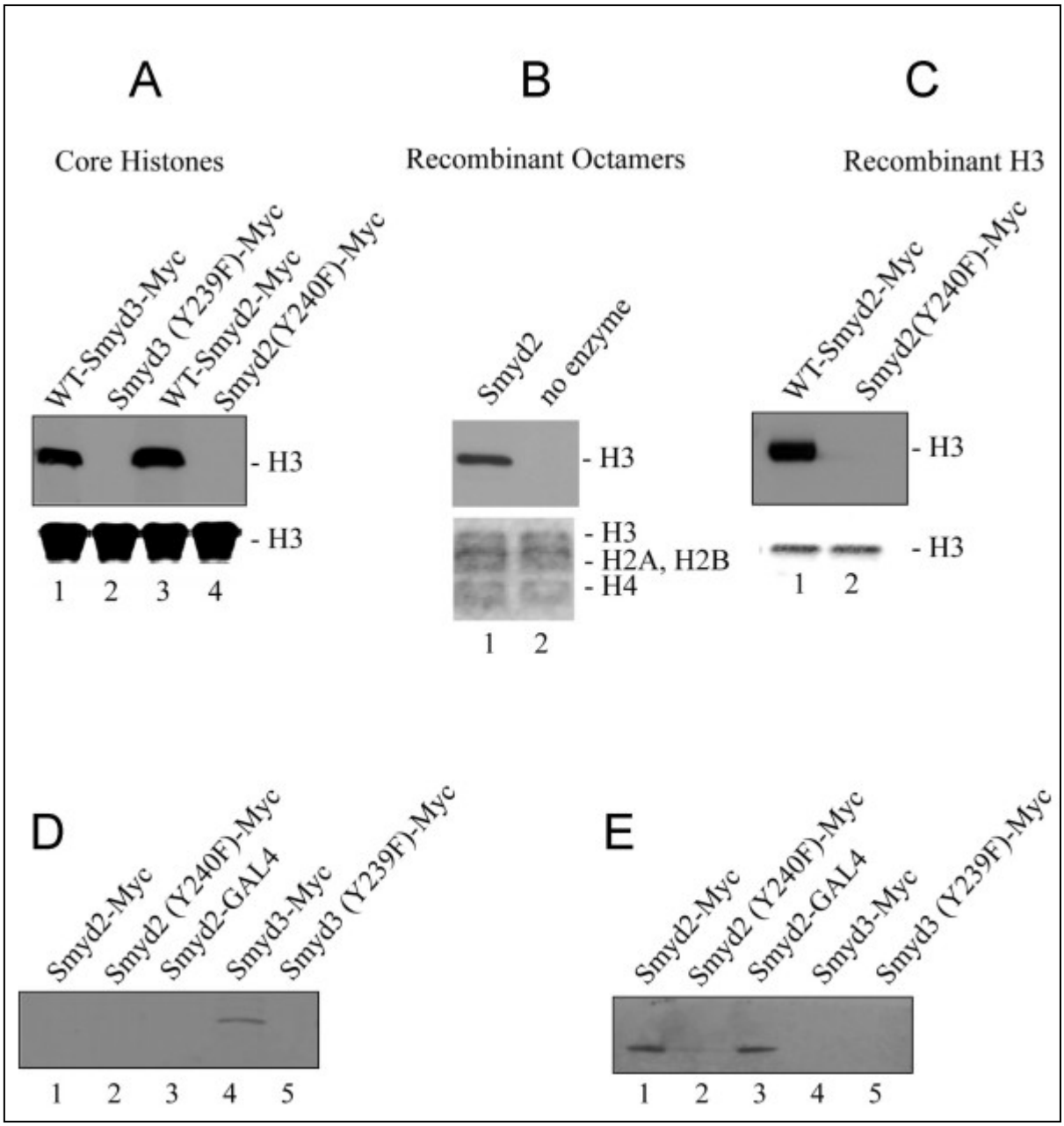

Figure 3.2: Smyd2 dimethylates histone H3, lysine 36. (A) Smyd2 methylates histone H3 in an *in vitro* histone methyl transferase (HKMT) assay using mixed histones from calf thymus as substrate. Fluorograms are shown in the upper panel; the 17 kD band, corresponding with Histone H3, is indicated; Coomassie stained SDS-PAGE gels were used to verify equal loading and are depicted in the lower panels. Lanes 1 and 3 show positive HKMT activity at H3 by myc tagged Smyd3 and myc tagged Smyd2, respectively. Lanes 2 and 4 indicate that neither the Smyd3 (Y239F) nor the Smyd2 (Y240F) catalytic mutants have HKMT activity. It is concluded that the HKMT activity of Smyd3 depends on Y239 and

Y240 for Smyd2. (B) Smyd2 methylates Histone H3 in an *in vitro* histone methyl transferase assay using recombinant octamers as substrate. Fluorograms are shown in the upper panel; the 17 kD band, corresponding with Histone H3, is indicated; Coomassie stained SDS-PAGE gels were used to verify equal loading and are depicted in the lower panels. Histone H3 was found methylated by Smyd2 using recombinant octamers as substrate in an *in-vitro* HKMT assay. (C) Smyd2 methylates histone H3 in an *in vitro* histone methyl transferase assay using recombinant histone H3 as a substrate. Fluorograms are shown in the upper panel; the 17 kD band, corresponding with histone H3, is indicated; Coomassie stained SDS-PAGE gels were used to verify equal loading and are depicted in the lower panels. Histone H3 was found methylated by Smyd2 using recombinant octamers as substrate in an *in-vitro* HKMT assay (lane 1). The catalytically defective mutant Smyd2 (Y240F) failed to methylate recombinant histone H3 (lane 2). It is concluded that the HKMT activity of Smyd2 depends on Y240. (D) Smyd2 does not dimethylate histone H3 at lysine 4 using recombinant histone H3 as a substrate in an *in-vitro* HKMT assay. Western results, using antibodies, specifically reactive with dimethylated histone H3, lysine 4, are shown; the 17 kD band, corresponding with histone H3, is indicated. Lanes 1 and 4 indicate that immunoprecipitated and myc-tagged Smyd3, but not myc-tagged Smyd2, dimethylates histone H3 at lysine 4. Lanes 2 and 5 show that neither Smyd2 (Y240F) nor Smyd3 (Y239F) dimethylate histone H3 at lysine 4. We conclude that Smyd2 does not dimethylate histone H3 at lysine 4. (E) Smyd2 dimethylates histone H3 lysine 36 using recombinant histone H3 as a substrate in an *in-vitro* HKMT assay. Western results, using antibodies, specifically reactive with dimethylated histone H3, lysine 36, are shown; the 17 kD band, corresponding with histone H3, is indicated. Lanes 1 and 3 indicate that Smyd2 dimethylates recombinant histone H3 at lysine 36, independent of the myc or Gal4 tag. The catalytically inactive mutant Y240F does not dimethylate recombinant histone H3 at lysine 36 (lane 2). Smyd3, as well as the catalytically defective mutant Y239F, do not dimethylate recombinant histone H3 at lysine 36 (lanes 4 and 5). We conclude that Smyd2 dimethylates recombinant histone H3 at lysine 36, whereas Smyd3 does not display this activity. (Adapted from Brown, et al. [100])

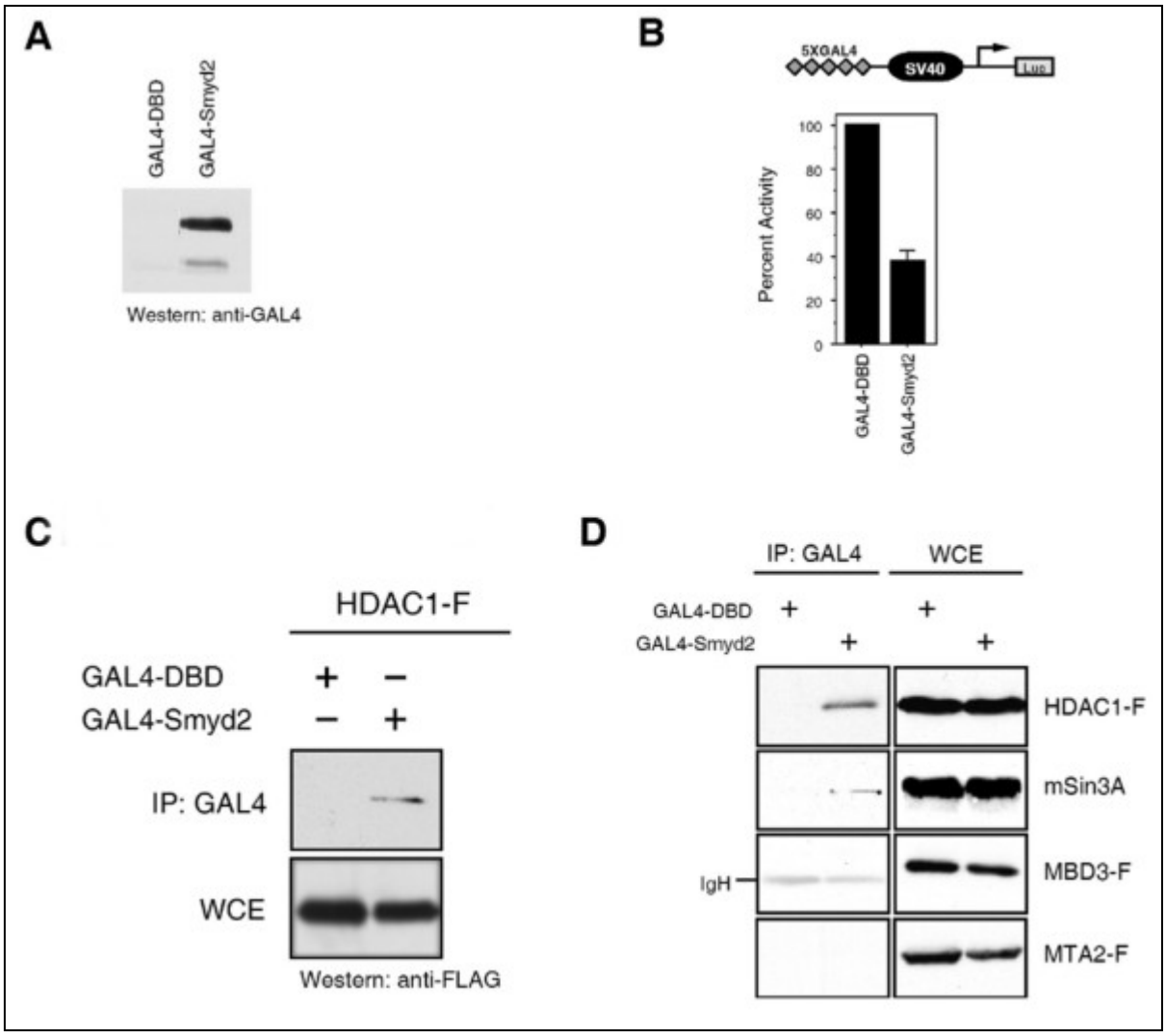

Figure 3.3: Smyd2 associates with the Sin3 repression complex and is involved in transcriptional repression. (A) Expression of GAL4-Smyd2 fusion protein in 293T cells. 293T cells were transfected with the constructs indicated and, 48 hours post transfection, whole cell lysate was prepared using RIPA buffer and subjected to western analysis using antibodies directed against the GAL4 tag. A reactive band was detected at the appropriate molecular weight (approximately 66 kD). Extracts from cells, transfected with the GAL4-DBD construct [17], served as negative control. (B) Smyd2 represses transcription of a luciferase reporter. Top panel: Schematic illustration of the reporter construct used. Bottom panel: 10T1/2 cells were transiently co-transfected with the 5XGAL4-SV40-luciferase reporter (1 µg) together with GAL4-DBD or GAL4-Smyd2 (2 µg each). Percent activity of the luciferase was determined in relation to GAL4-DBD. Smyd2 significantly represses

the transcription of a luciferase reporter in 10T1/2 cells. (C) Smyd2 associates with HDAC1. Exponentially grown 293T cells were transiently co-transfected with GAL4-DBD or GAL4-Smyd2, together with Flag-tagged HDAC1 (HDAC1-F). Whole cell RIPA extracts were immunoprecipitated using an anti-GAL4 antibody and immunoblots were probed with an anti-FLAG antibody. As shown here, Smyd2 associates with HDAC1. RIPA whole cell extracts from GAL4-DBD transfected cells [17] served as negative control. Equal protein amounts in the immunopreciptation assays were demonstrated by analysis of 5% input using anti Flag antibodies. (D) Smyd2 interacts with the Sin3A but not the NuRD complex. Exponentially grown 293T cells were transfected with the constructs indicated and, 48 hours post transfection, whole RIPA lysate was prepared. Antibodies directed against GAL4 were used for immunoprecipitation, followed by western analysis using the antibodies indicated. Smyd2 associates with HDAC1 and Sin3A but not with the components of the NuRD complex, MBD3 or MTA2. (Adapted from Brown, et al. [100])

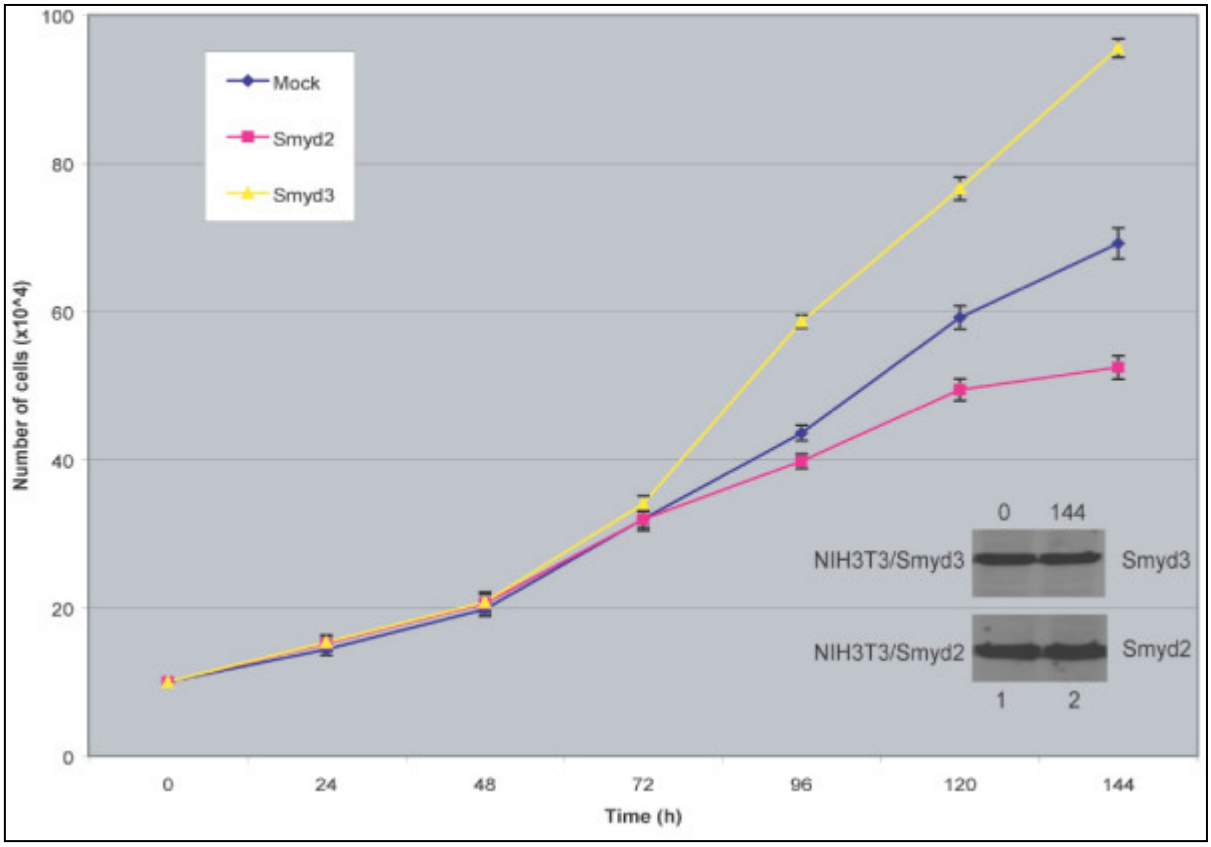

Figure 3.4: Smyd2 suppresses NIH3T3 cell proliferation. NIH3T3 cells were transfected with plasmids encoding myc-tagged Smyd2 or myc-tagged Smyd3. Cells transfected with the empty expression construct (Mock) served as control. All cells were monitored by cell counting using trypan blue exclusion. The inserts show the level of expression of Smyd2-myc and Smyd3-myc at 24 and 144 hours post transfection, demonstrating similar levels of ectopically introduced proteins in the NIH3T3 cell lines used. Whereas ectopically introduced Smyd3 enhanced the proliferation, Smyd2 displayed a negative effect on the growth rate of NIH3T3 cells. (Adapted from Brown, et al. [100])

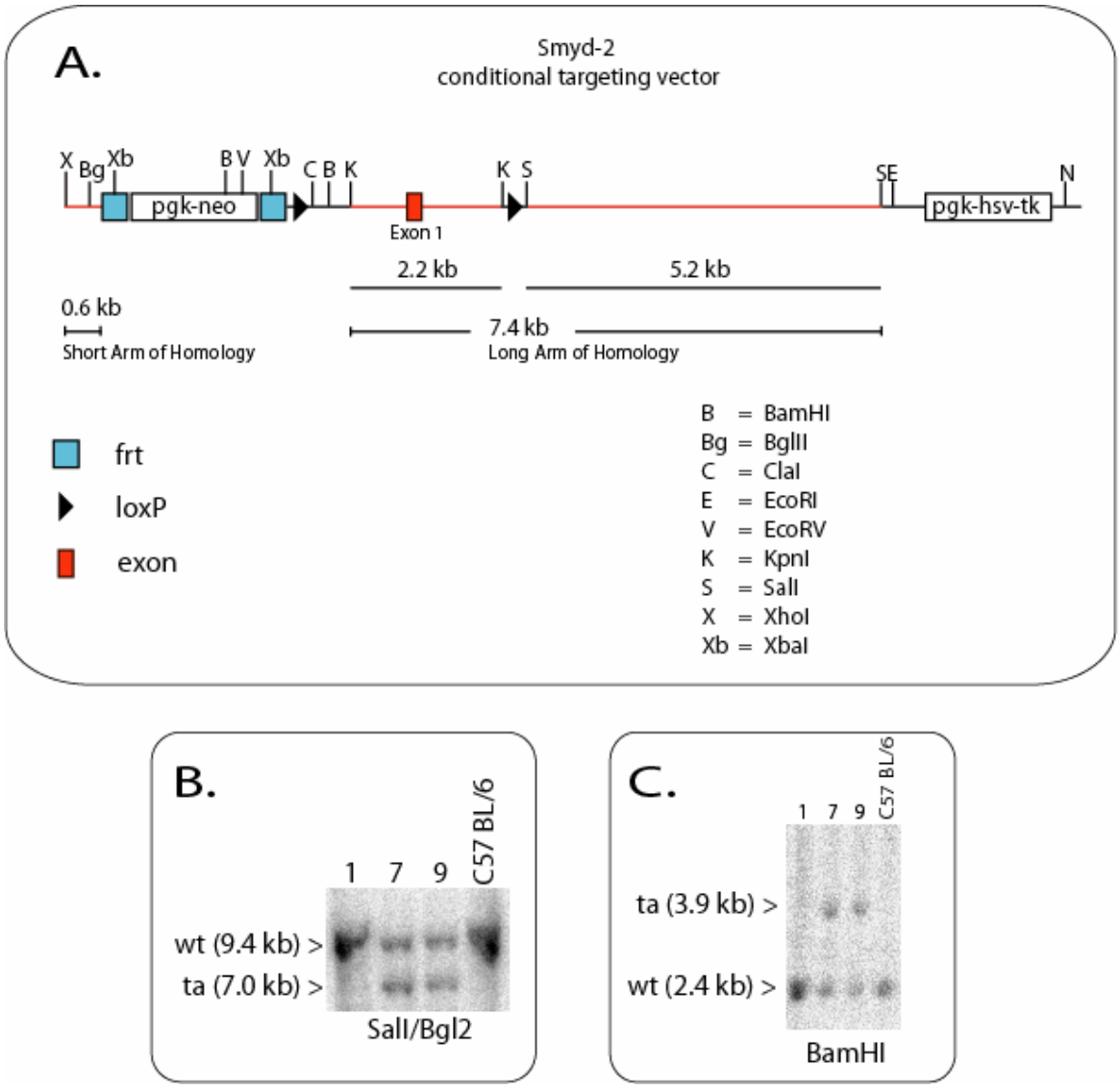

Figure 3.5: Strategy for the conditional targeting of the Smyd2 gene. (A) Schematic illustration of the targeting construct. The targeting vector has a short (0.6kb) and a long (7.4 kb) arm of homology, a neomycin cassette (pgk-neo) for positive selection, and a thymidine kinase cassette (pgk-hsv-tk) for negative selection. Two site-specific recombination sites are employed in vivo. (1) Flp recombinase to delete the neo marker from the mouse germline, and (2) Cre recombinase to conditionally delete Smyd2 in selected tissues. Two loxP sites flank the region to be deleted. Enzyme restriction sites are indicated. (B and C) Characterization of successfully targeted embryonic stem (ES) cells by Southern analysis. Southern analyses were performed across the long (panel B) and the

short (panel C) arms of homology of clones that have experienced homologous recombination at the Smyd2 locus. A size difference allows the separation of targeted allele (ta) and wild type (wt) allele. Fragment size is indicated in kilo base pairs (kb). The restriction enzymes used for analyses are indicated at the bottom of panels B and C.

4. PROMISCUITY OF SMYD3-MEDIATED METHYLATION

4.1 Abstract

Aberrant expression of the histone H3-lysine 4 (H3K4)-specific methyltransferase, Smyd3, has been implicated in colorectal, hepatocellular, and breast cell carcinogenesis. Here, Smyd3 is also shown to target histone H4, lysine 20 (H4K20). However, over-expression of Smyd3 in fibroblasts results in global reduction of trimethylation at H4K20 and this is accompanied by a striking increase in cell proliferation. As the methylation of H3K4 and H4K20 are normally associated with conflicting biological roles, I predict that these differential activities of Smyd3 are manifest under spatially and/or temporally distinct conditions, in the presence of different associating complexes, thereby resulting in effects that may be antagonistic of one another.

4.2 Background

In the developed world, colorectal carcinoma (CRC) is the primary source of malignancy associated with cancer deaths [106, 143, 144]. Worldwide, hepatocellular carcinoma (HCC) accounts for the predominant cause in cancer mortalities and the occurrence of this type of tumor is increasing at an alarming rate in the United States [145]. As changes in chromatin accessibility appear to play a central role in the progression of such neoplasias, significant effort is being

invested toward understanding the molecular mechanisms that govern the dynamic architecture of chromatin [146, 147].

Cellular characteristics are retained through epigenetic mechanisms that impart heritable blueprints for gene expression [107, 148]. These histone codes are commonly expressed through post-translational modifications at specific histone residues in a chronologically-sensitive manner [60, 149]. Common modifications include methylation, acetylation, phosphorylation, and ubiquitination [149]. The transcriptional interpretation of these programmed events corresponds with distinct biological outcomes [107].

Smyd3 has been identified as a H3K4-specific methyltransferase [106]. This modification often acts in concert with alterations in DNA methylation to impart permanent specificity of cell-type facilitated through long-term gene expression [108]. The findings that Smyd3 is substantially upregulated in most CRCs [150], HCCs [151], and breast cancer tissues [152] support a paradigm in which aberrant expression of chromatin-modifying enzymes, leading to a disturbance in established epigenetic patterns, can ultimately result in tumorigenesis.

Through microarray analyses, 80 genes have been identified that display altered gene expression in the presence of Smyd3 [106]. Notably, one of these is Nkx2.8, a homeobox gene that exhibits upregulation in hepatocellular malignancies [153]. Other affected genes include cell cycle regulators, oncogenes, and several that are critical in developmental processes [106]. In

concert with the idea that Smyd3 is a transcriptional activator, it forms a complex with RNA polymerase II and it was also shown to bind DNA directly by way of a sequence found in the promoter of Nkx2.8 [106]. These findings not only provide targets for the H3K4 enzymatic activity of Smyd3 but they also implicate two methods for its direct interaction with those genes (Fig. 4.1 A & B).

Figure 4.2 illustrates a likely paradigm in which the aberrant expression of Smyd3 may result in the upregulation of otherwise silent genes such as Nkx2.8. This can disrupt the balance of cellular gene expression, ultimately resulting in transformation. This idea is corroborated not only indirectly, by the findings that Smyd3 is over-expressed in CRCs, HCCs, and breast cancers, but also more directly by the effect that Smyd3 has on cell proliferation. The over-expression of Smyd3 in NIH3T3 cells accelerates propagation while siRNA knockdown of Smyd3 reverses these effects [106].

4.3 Results

4.31 Smyd3 enzymatic activity

As mentioned above, Smyd3 had previously been identified as a histone H3K4-specific methyltransferase. However, in their ground-breaking publication, Hamamoto et al. failed to examine any potential substrates for Smyd3, beyond recombinant histone H3 [106]. Therefore, to examine the possibility that Smyd3 may have more than one histone substrate, I examined its ability to methylate

mixed histones isolated by acid extraction from HeLa cells. Histone methylation was tested after incubation of Smyd3 with S-adenosyl-L-[methyl-^{3}H] methionine (SAM) and the mixed histones from HeLa cells as a substrate. A 17kD band corresponding to ^{3}H-labelled H3 was visible in the lane containing Smyd3 on the fluorogram (Fig. 4.3A), confirming that Smyd3 has intrinsic H3-specific activity. Surprisingly however, a more intense band corresponding with a smaller molecular weight was also observed, indicating that in addition to methylating H3, Smyd3 also methylates one or more of the diminutive histones (H2A, H2B, and/or H4).

Smyd3 HKMT activity was next tested separately on recombinant histone H2A, H2B, H3, and H4 substrates. Smyd3 methylated all of the histones to various degrees (Fig. 4.3B). As H4 appeared to be the most vigorously methylated substrate, I concentrated my efforts on confirming the activities of Smyd3 on H4. A tyrosine in the C-terminal region of the core SET domain is conserved among catalytically active SET domain proteins (Fig. 4.3B). Therefore, to confirm the authenticity of Smyd3 as a SET-dependent HMTase capable of methylating both H3 and H4, a catalytic mutant of Smyd3 (Smyd3-Y239F-Myc, see chapter 3.4) was analyzed for its ability to methylate H3 and H4 as compared with wild-type (Smyd3-myc). Smyd3-Y239F-Myc displayed absolutely no HKMT activity on either H3 or H4 (Fig. 4.3C). However, the wild-type Smyd3-Myc displayed activity in the presence of both H3 and H4 (Fig. 4.3C). Therefore, I concluded that Smyd3 has SET domain-dependent, intrinsic HKMT activity for both H3 and H4 substrates.

Given that the only documented mammalian site of lysine methylation on histone H4 is at H4K20 [154], I tested whether H4K20 is the residue targeted by Smyd3. Histone methyltransferase assays were performed using recombinant H4, and specificity was determined by western blotting using antibodies against monomethyl H4K20 and trimethyl H4K20. Smyd1B was included as a negative control. No band corresponding with H4 was detected when reactions were probed with anti-monomethyl H4K20 (Fig. 4.4B). However, when reactions were probed with anti-trimethyl H4K20, a band corresponding to H4 was observed with Smyd3, but not with Smyd1 (Fig. 4.4C.). Therefore, it is concluded that Smyd3 trimethylates H4K20.

To strengthen these findings, I performed a series of proximity bead histone methyltransferase assays using Smyd3 with four different H4 peptides varying only with regard to their methylation state at K20. Trimethylated H4K20 was included as a negative control. According to scintillation counts, Smyd3 activity was strongest on a dimethylated H4 peptide (Fig. 4.4A). This is in accord with the H4K20 antibody results. Modest activity was also observed on a monomethylated H4 peptide (Fig. 4.4A). However, scintillation counts demonstrated that non-methylated H4 peptides are not strongly acted upon by Smyd3 (Fig. 4.4A).

4.32 Effects of Smyd3 on global methylation patterns

Cell differentiation is driven by the progressive regulation of gene expression patterns that favor distinct cell types [155]. Histone modifications govern many of these regulatory processes by their effects on chromatin accessibility and the recruitment of either transcriptional-activation or -repression complexes [156]. For example, global levels of histone acetylation and H3K4 methylation, which are most often associated with transcriptionally active chromatin, decline during the differentiation of embryonic stem (ES) cells [157]. Reciprocally, the levels of H4K20 and H3K9 trimethylation, which are most often connected with gene silencing, are elevated during the differentiation of ES cells [158]. These findings indicate that global transcriptional competence becomes more constrained during cell differentiation. Conversely, the re-initiation of pluripotency in somatic cells requires the re-establishment of histone modifications that are conducive to the formation of transcriptionally active euchromatin, including elevated levels of global acetylation and H3K4 methylation [159].

Recent advances in cancer research make it increasingly evident that oncogenesis often arises from aberrations in patterns of gene expression that define and maintain cellular memory [160]. In fact, almost all classes of cancer involve deviant epigenetic modifications that impart heritable changes necessary for tumorigenesis and metastatic progression [161-164]. Studies showing that the H3K4-specific methyltransferase acitivity of Smyd3 is involved in several types of cancer [106, 152] are in accord with other research that correlates

increases in global methylation at H3K4 with augmented cell proliferation [160-162]. However, my recent findings, demonstrating that Smyd3 is also competent in the trimethylation of H4K20, complicate this matter. Although Smyd3 has been shown to methylate H3K4 near the promoters of specific genes which it is known to upregulate, the effect of Smyd3 on global methylation at H3K4 has not been evaluated. To clarify the paradoxical specificities of Smyd3 at H3K4 and H4K20, I analyzed the global methylation patterns associated with the expression of Smyd3 in fibroblasts.

To determine if the over-expression of Smyd3 affects global methylation patterns in fibroblasts, I conducted a series of transfection experiments in which I over-expressed wild type Smyd3-Myc or Smyd3 (Y239F)-Myc in NIH3T3 cells. After 36 hours, I isolated histones from the transfected cells by acid extraction and conducted a series of western blots on the resulting fractions using the following antibodies: anti-trimethyl H3K4; anti-trimethyl H3K9, and anti-trimethyl H4K20. Levels of H3K4 and H3K9 trimethylation appeared unchanged in the presence of either wild type Smyd3 or its catalytic mutant, relative to that observed on histones from mock transfected cells (Fig. 4.5B). However, histones from cells transfected with wild type Smyd3 exhibited a marked **decrease** in H4K20 trimethylation when compared to histones from mock transfected cells (Fig. 4.5A). Therefore, I concluded that over-expression of Smyd3 results in a significant decrease in the global methylation of H4K20 in NIH3T3 cells. Furthermore, over-expression of Smyd3 does not affect global methylation at H3K4 or H3K9 in NIH3T3 cells.

4.33 Production of a Smyd3 polyclonal antibody

As the methylation of H3K4 and H4K20 are normally associated with conflicting biological roles, it seems reasonable to speculate that the differential activities of Smyd3 are manifest under spatially and/or temporally distinct conditions, in the presence of different associating complexes, thereby resulting in effects that may be antagonistic of one another. Therefore, to facilitate our investigation of endogenous Smyd3 expression and its interacting partners/complexes, I produced a polyclonal antibody that specifically reacts with Smyd3.

Recombinant human Smyd3 was commercially produced using a baculoviral infection system in which an ovarian cell line from S*podoptera frugiperda* served as the host. I generated Smyd3 polyclonal antibodies by immunizing New Zealand White rabbits four times each with purified Smyd3. Rabbit serum was collected before immunization as a negative control. The specificity of the Smyd3 antibody was tested by comparing its reactivity on western blots arrayed with lysates from 293T cells over-expressing Smyd3 fusion proteins versus lysates of 293T cells over-expressing Smyd1 and Smyd2 fusion proteins (Fig. 4.6A & B). At a dilution of 1:2000, the Smyd3 polyclonal antiserum was highly reactive against Smyd3 fusion proteins but completely inert toward Smyd1 and Smyd2 fusion proteins. Smyd3 polyclonal antibody was then used to analyze the expression of Smyd3 proteins in adult murine tissues. At a 1:2000

dilution, Smyd3 protein was detected in adult heart and liver tissues but not in spleen (Fig. 4.6C). Finally, the Smyd3 antibody was used to determine if Smyd3 is highly expressed in common cell lines. Smyd3 was detectable in two T-cell lines (Jurkat and Molt-13; data not shown). These tissue and cell lysate western blots correspond with previous northern blots indicating that Smyd3 transcripts are expressed in thymus, liver, and cardiac tissues but not in spleen.

4.4 Discussion

Lysine-rich N-terminal tails project from within the histone core of chromatin and are the targets of a variety of post-translational modifications [165]. These alterations impact the degree of chromatin condensation which, in turn, regulates transcriptional activity [166]. Histone methylation is one such modification and it has been shown to be critical in the establishment of heritable patterns of gene expression [167]. Numerous studies have associated aberrant histone methylation with the induction and progression of human disease [168]. For this reason, there is growing interest in the identification of the enzymes responsible for histone methylation and the ramifications of their potential for deviant activity [169]. Smyd3 has, therefore, emerged as the subject of intense research for its role as a histone methyltransferase involved in the induction of hepatocellular, colorectal, and breast cancers [106, 152].

The correlation between Smyd3 over-expression in mature tissues and tumorigenesis prompted our functional analysis of Smyd3. To this end, we have

determined that Smyd3 is promiscuously competent in the methylation of histone residues. In addition to its previously identified target at H3K4, Smyd3 robustly trimethylates H4K20 *in vitro*. The biological effects of Smyd3 appear to be tissue-specific as over-expression of Smyd3 in fibroblasts results in a global reduction of trimethylation at H4K20. Therefore, we have produced a highly specific polyclonal antibody against Smyd3 to facilitate our investigation of endogenous Smyd3 expression and its interacting partners/complexes.

4.41 Smyd3 enzymatic activity

Using recombinant human Smyd3, I confirmed the authenticity of Smyd3 as a SET-dependent histone methyltransferase, capable of methylating histone H3. In addition, I found that Smyd3 is also competent as a catalyst of histone H4 methylation. Although dual-specificity methyltransferases have been previously identified [170], they are certainly rare. SET9 for example, is known to methylate H3K4 [171] as well as lysine 372 of p53 [172]. Likewise, the ability of enzymes to methylate more than one **histone** residue is exceptionally rare [173]. One such enzyme, *Drosophila* Ash1, is recognized for its capacity to methylate H3K4, H3K9, and H4K20. However, its mammalian ortholog is specific only for H3K36 [174]. In the case of *Drosophila* Ash1, the multiple methylation sites have been demonstrated to work synergistically to induce transcriptional activation by recruiting chromatin remodeling factors and preventing the binding of transcriptional repressors [173]. Likewise, H3K4 methylation by Smyd3 has been

linked to transcriptional activation and Smyd3 is known to interact with RNA polymerase II and HELZ, both of which are associated with actively transcribed euchromatin [106]. However, we have also shown that Smyd3 interacts with the nuclear receptor co-repressor, N-CoR, and the Sin3 transcriptional repression complex (Fig. 4.7). Therefore, although it is reasonable to speculate that the dual-specificities of Smyd3 function synergistically in a manner analogous to *Drosophila* Ash1, it is just as likely that each specificity functions separately. In the latter paradigm, one can imagine that the differential activities of Smyd3 are manifest under spatially and/or temporally distinct conditions, in the presence of different associating complexes, thereby resulting in effects that may be antagonistic of one another. Indeed, the contradictory physiological consequences of H3K4 trimethylation versus H4K20 trimethylation parallel the distinct biological roles associated with RNAPII transcriptionally active complexes versus the N-CoR and Sin3 transcriptional repression complexes. In the context of pharmacologically impairing the activity of Smyd3 in a therapeutic intervention against cancer, the answers to these uncertainties are paramount. Specifically, it must be known whether the global inhibition of Smyd3 *in vivo* will result in disparate outcomes on a tissue-type and -age specific basis.

4.42 Potential role for Smyd3-mediated reduction of global trimethylation at H4K20

Using antibodies against several common sites of histone methylation, I determined that over-expression of Smyd3 reduces global trimethylation at H4K20 in fibroblasts. Normal methylation at this site is required for maintaining constitutive heterochromatin and overall genomic stability. A potential mechanism might involve the tumor suppressor, Retinoblastoma (Rb). Rb is known to directly interact with the H4K20 trimethylating enzymes, Suv4-20h1 and Suv4-20h2. Fibroblasts that lack Rb show decreased trimethylation at H4K20, accompanied by striking genomic instability [175, 176]. The C-terminal region of Rb contains the consensus Smyd interacting motif, PXLXP. Thus, it will be informative to determine if Rb is a direct methylation target of Smyd3. If so, it will also be revealing to examine whether Smyd3-mediated methylation of Rb affects the ability of Rb to interact with the Suv-20 enzymes. Such a finding would indicate that aberrant methylation of Rb by Smyd3 may be responsible for the decrease in global trimethylation at H4K20 in Smyd3-over-expressing fibroblasts.

4.5 Conclusion

I conclude that Smyd3 is a multiple-specificity histone methyltransferase. Though the over-expression of Smyd3 in NIH3T3 cells results in global reduction of trimethylation at H4K20, accompanied by a striking increase in cell proliferation, the effects of Smyd3 in other cell types may vary. Current

mutational analyses of Smyd3 will undoubtedly reveal additional features that distinguish Smyd3 from other methyltransferases by defining its catalytic parameters, mechanisms of specificity, and protein interaction regions.

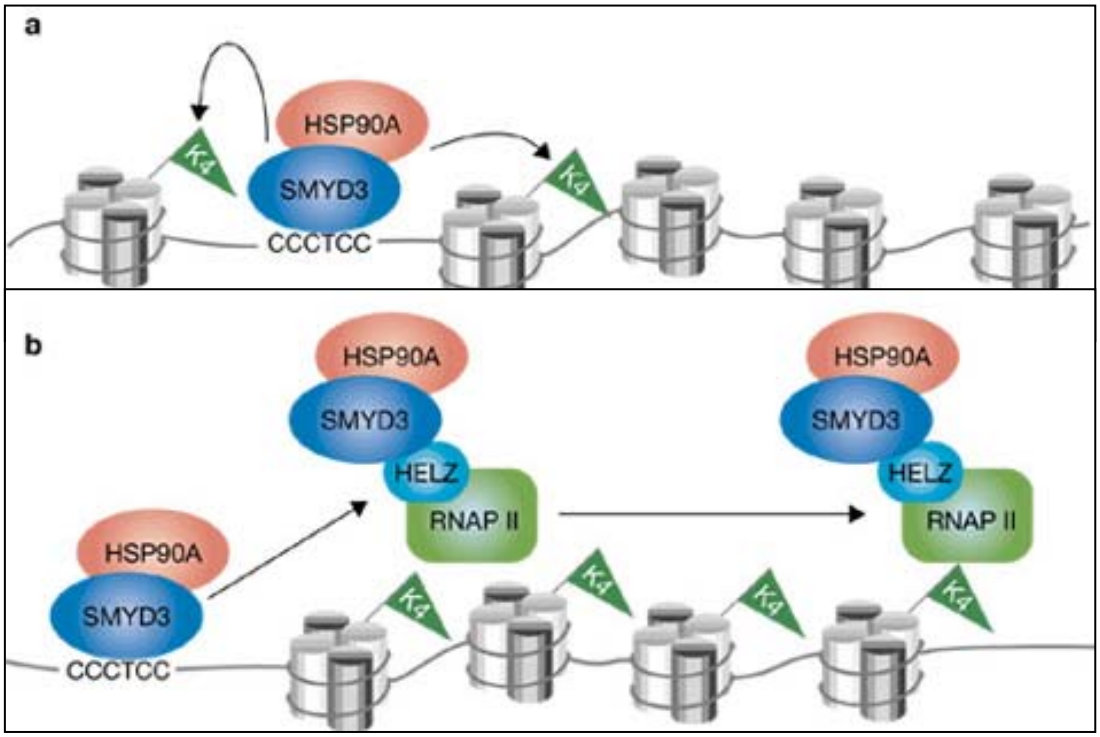

Figure 4.1: Model for Smyd3 methylation of H3K4. (a) Smyd3 is directed to histones via recognition of a nearby Smyd3 binding site (5'-CCCTCC-3' motif). Upon recruitment, Smyd3 specifically methylates H3K4 of proximal histones. Interaction of Smyd3 with Hsp90 is known to enhance Smyd3-mediated methylation of H3K4. (b) Alternatively, recruitment of Smyd3 to its DNA binding element may facilitate its subsequent interaction with RNA polymerase II through its recruitment by the RNA helicase, HELZ. Smyd3 could thereby progressively methylate H3K4 while it remains associated with the RNA polymerase II complex. (Adapted from Sims & Reinberg [98])

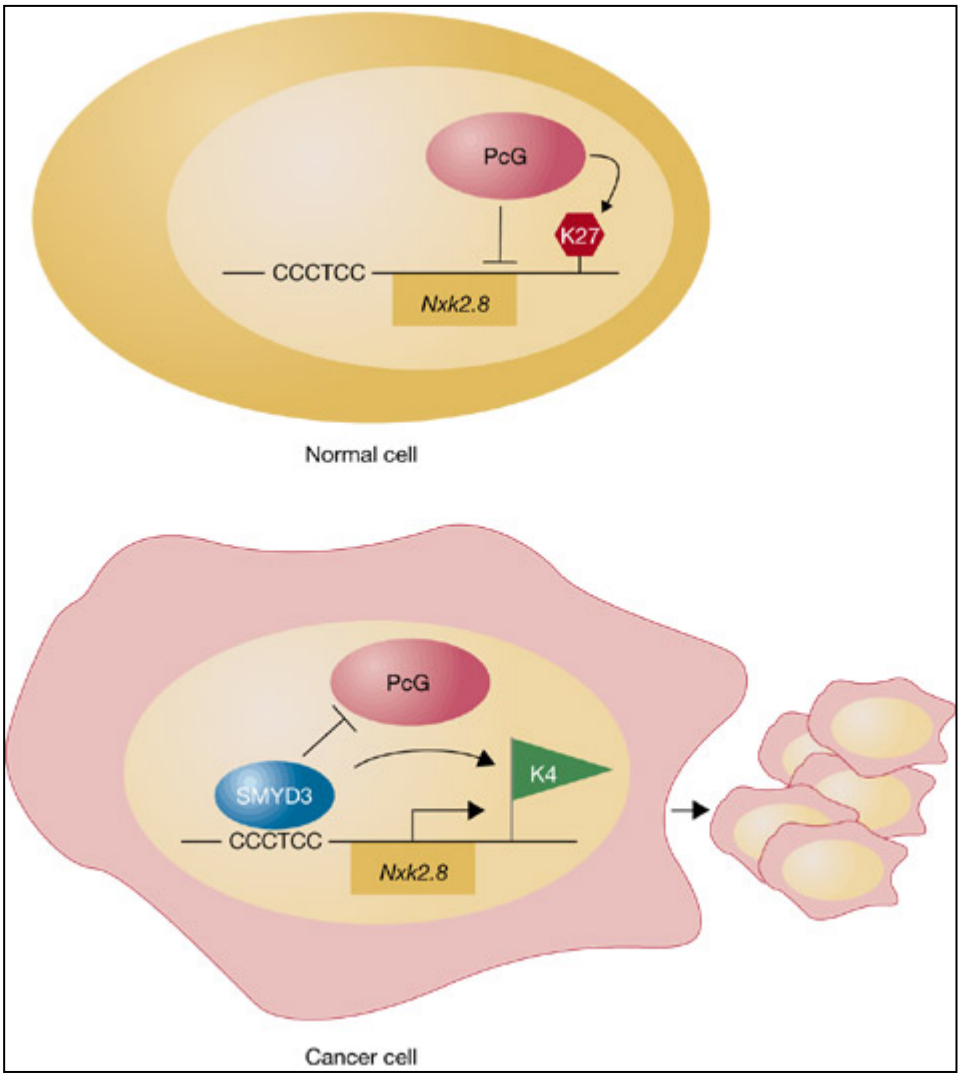

Figure 4.2: Potential role of Smyd3 in tumorigenesis. Tissue-specific genes are often preserved in a transcriptionally silent state by the Polycomb group (PcG) of proteins (top). When aberrantly expressed, Smyd3 may interact with its target genes, such as *Nxk2.8*, and out-compete the PcG-mediated silencing (bottom). Transcriptionally repressive methyl marks, such as H3K27, may thereby be altered to H3K4 by Smyd3, resulting in transcriptional activation and eventually, tumorigenesis. (Adapted from Sims & Reinberg [98])

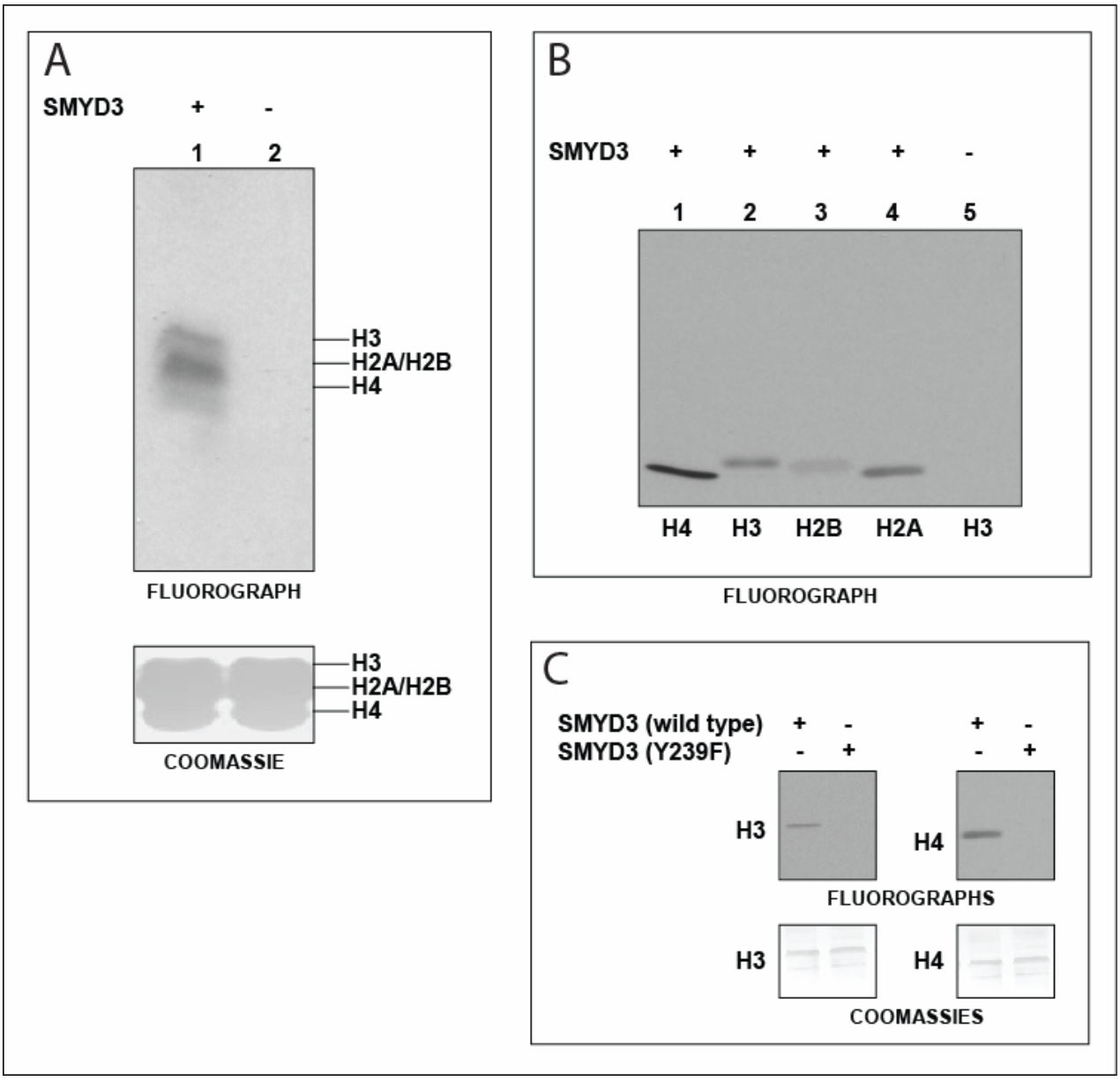

Figure 4.3: Promiscuity of Smyd3-mediated histone methylation. (A) Smyd3 methylates histone H3 in addition to one or more of the diminutive histones (H2A, H2B, and/or H4) in an *in vitro* histone methyl transferase (HKMT) assay using mixed histones from HeLa cells as substrate. Fluorograph is shown in the upper panel; the 17 kD band, corresponding with Histone H3, is indicated; several smaller bands, presumably H2A/H2B and/or H4 are also indicated. A coomassie stained SDS-PAGE gel was used to verify equal loading and is depicted in the lower panel. (B) Smyd3 methylates histones H2A, H2B, H3, and H4 in an *in vitro* HKMT assay using recombinant histones, as indicated, for substrate. Fluorograph is shown and the bands corresponding with each histone

are indicated. (C) Wild type Smyd3, but not a catalytic point mutant (Y239F), methylate H3 and H4 in an *in vitro* HKMT assay using recombinant histones, as indicated, for substrate. Fluorographs are shown in the upper panels; bands corresponding with H3 (left) and H4 (right) are indicated. Coomassie stained PVDF membranes were used to verify equal loading and are depicted in the lower panels.

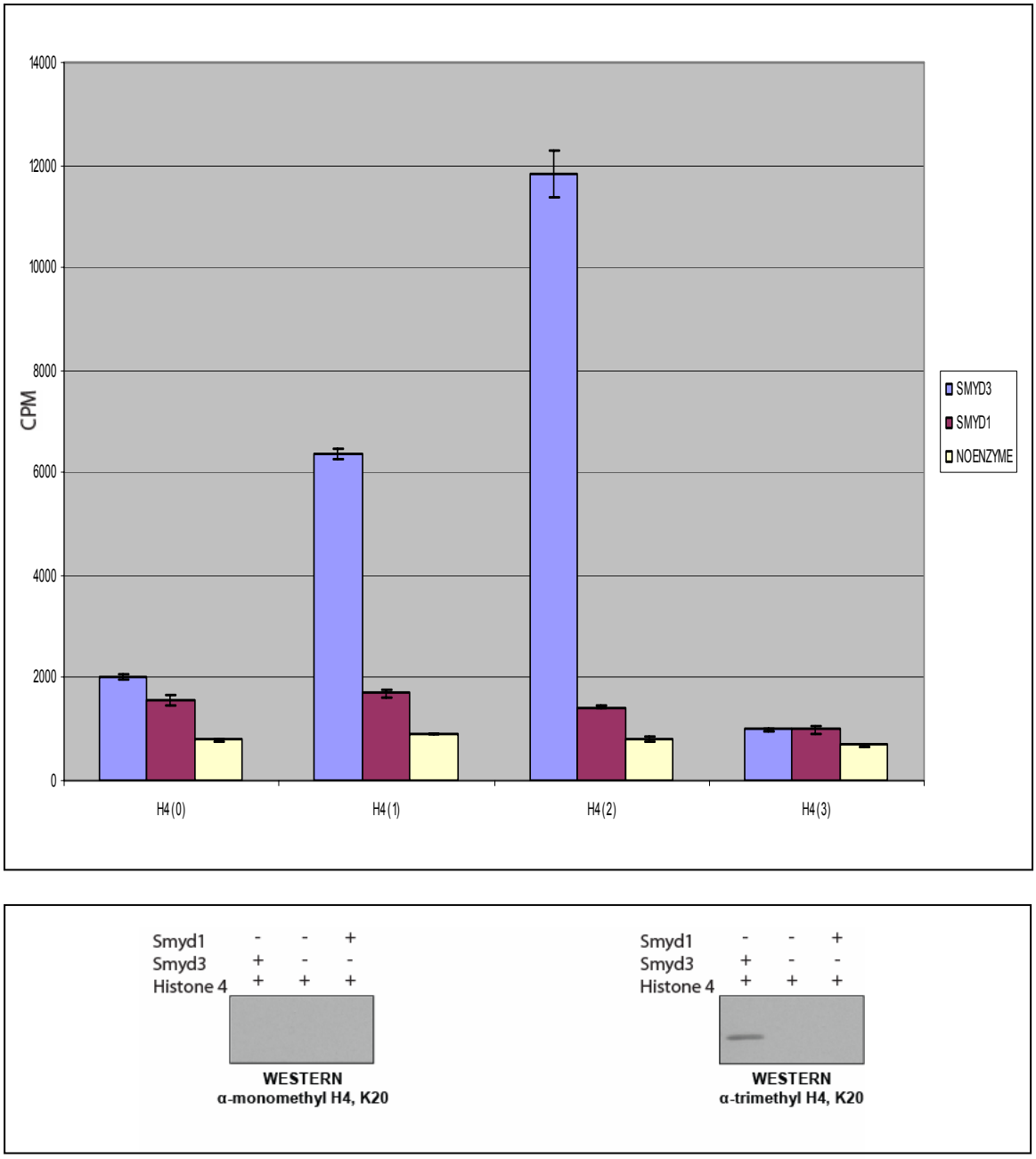

Figure 4.4: Smyd3 trimethylates H4K20. (A) Smyd3 methylates mono-methylated [H4(1)] and dimethylated [H4(2)] peptides in an *in vitro* histone methyltransferase (HKMT) assay using H4 peptides, representing all four states of methylation, as substrates. Degree of methylation was measured by scintillation counting and the results are graphically displayed in counts per minute (CPM). (B) Smyd3 does not monomethylate histone H4 at lysine 20

using recombinant histone H4 as a substrate in an *in vitro* HKMT assay. Western results, using antibodies specifically reactive with monomethylated histone H4, lysine 20, are shown. (C) Smyd3 trimethylates H4K20 in an *in vitro* HKMT assay using recombinant H4 as substrate. Western results, using antibodies specifically reactive with trimethylated H4K20, are shown.

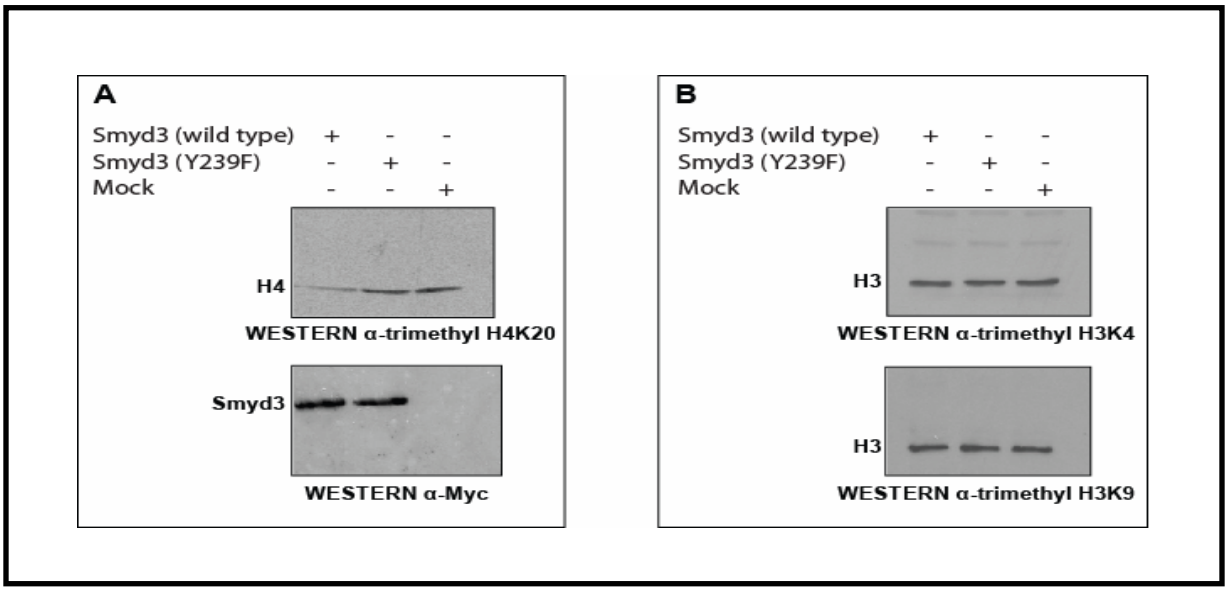

Figure 4.5: Over-expression of Smyd3 in fibroblasts results in global reduction of trimethylation at H4K20. (A) NIH3T3 cells were transfected with plasmids encoding Smyd3-Myc or Smyd3 (Y239F)-Myc. Cells, transfected with the empty expression construct (Mock), served as control. Histones were isolated by acid extraction 36 hours after transfection and antibodies were used to determine global states of methylation. Cells over-expressing wild type Smyd3 exhibited a markedly decreased level of trimethylation at H4K20 as determined by western results, using antibodies specifically reactive with trimethylated histone H4K20 (upper panel). The lower panel shows the levels of expression of Smyd3-Myc and Smyd3 (Y239F)-Myc at 36 hours post transfection. (B) NIH3T3 cells were transfected with plasmids encoding Smyd3-Myc or Smyd3 (Y239F)-Myc. Cells, transfected with the empty expression construct (Mock), served as control. Histones were isolated as described above and antibodies were used to determine global states of methylation. Cells over-expressing wild type Smyd3 did not exhibit any marked difference in levels of H3K4 trimethylation (upper panel) or H3K9 trimethylation (lower panel), relative to Smyd3 (Y239F) or the Mock control, as determined by western results, using antibodies as indicated.

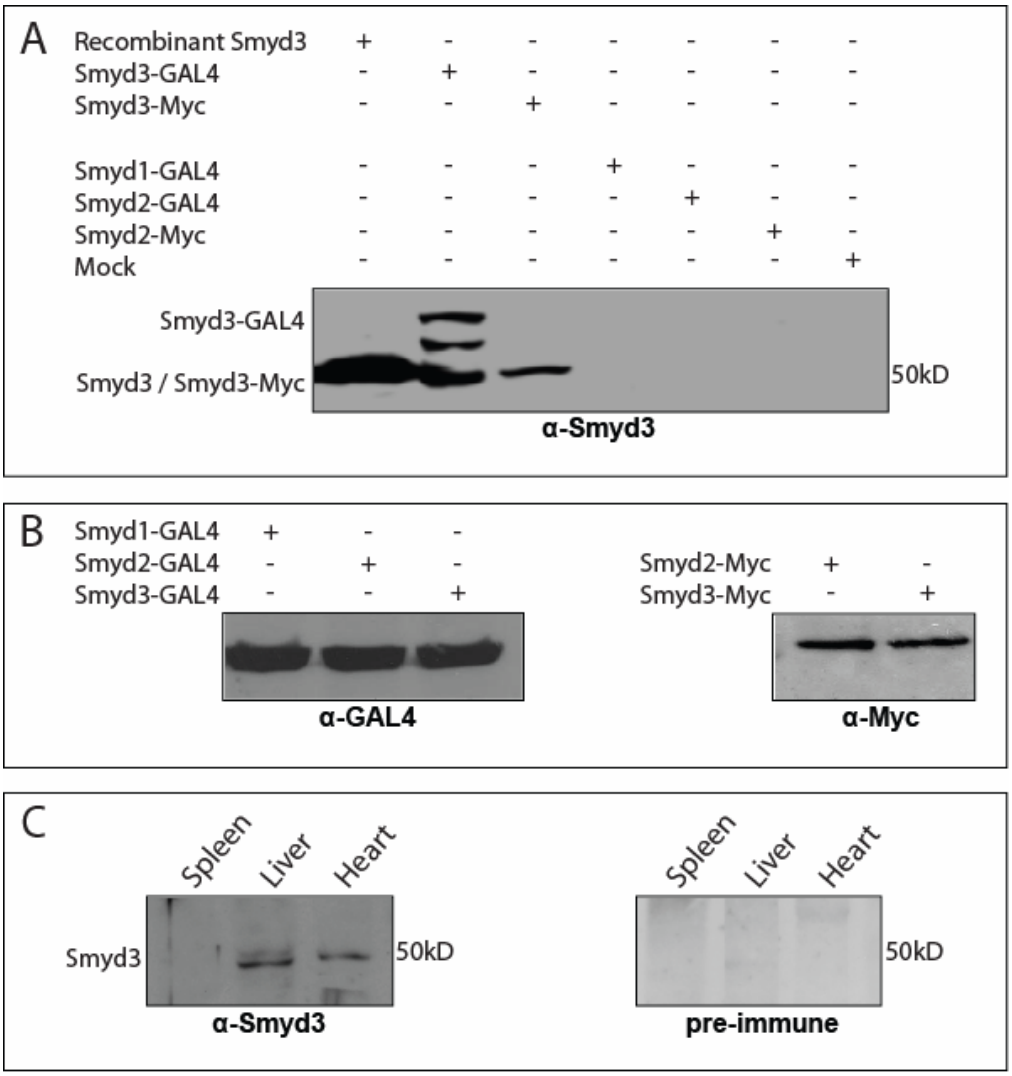

Figure 4.6: Characterization of Smyd3 polyclonal antiserum. (A) 293T cells were transfected with plasmids encoding Smyd1, Smyd2, and Smyd3 fusion proteins (as indicated). At a dilution of 1:2000, the Smyd3 polyclonal antiserum was highly reactive against whole cell lysates containing Smyd3 fusion proteins but completely inert toward whole cell lysates containing Smyd1 or Smyd2 fusion proteins. Recombinant Smyd3 was included as a positive control. (B) Fractions of the whole cell lysates described in (A) were probed with either α-Myc or α-GAL4 antibodies to confirm the expression of each fusion protein. (C) Spleen, liver, and cardiac tissues were harvested from adult mice and 100μg whole cell extracts were probed with Smyd3 antiserum. Smyd3 protein was detected in liver and cardiac tissues but not in the spleen (left panel). Pre-immune serum was included as a negative control (right panel).

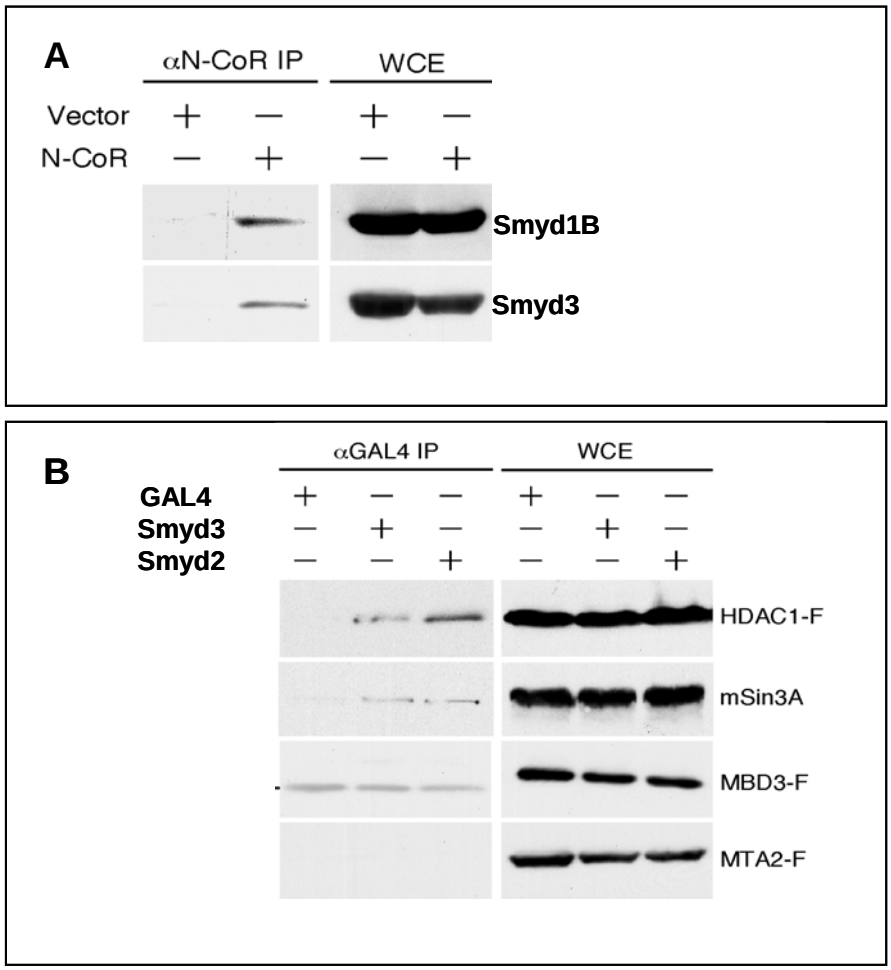

Figure 4.7: Smyd3 associates with N-CoR and components of the Sin3 repression complex. (A) The nuclear receptor co-repressor N-CoR has been shown to associate with the MYND domain-containing proteins ETO and BS69 *in vitro* and *in vivo* [107, 108]. N-CoR has also been shown to interact with the Sin3 complex, as well as the class I and class II HDACs. Since Smyd proteins associate with the Sin3 repression complex (B, [100]), we hypothesized that N-CoR participates in the association of Smyd proteins with class I and class II HDACs. 293T cells were transfected with the constructs indicated and, 48 hours post-transfection, whole RIPA lysates were prepared. Antibodies directed against N-CoR were used for immunoprecipitation, followed by western analysis using the antibodies indicated. Smyd1B and Smyd3 are shown to associate with N-CoR. (B) Smyd2 and Smyd3 interact with the Sin3A but not the NuRD complex. 293T cells were transfected with the constructs indicated and, 48 hours post transfection, whole RIPA lysates were prepared. Antibodies directed against

GAL4 were used for immunoprecipitation, followed by western analysis using the antibodies indicated. Smyd2 and Smyd3 associate with HDAC1 and Sin3A but not with the components of the NuRD complex, MBD3 or MTA2 (IgH bands are visible in the α-GAL4 lanes from the MBD3 IP experiment).

5. SMYD FAMILY CHARACTERIZATION

5.1 Abstract

The SMYD Family is a group of SET and MYND domain-containing transcriptional regulators that function, at least partly, through histone modifications. In spite of their similar architecture and enzymatic mechanisms, Smyd proteins induce dramatically disparate outcomes on cell proliferation. In the course of studying individual Smyd family members (chapters 3 and 4), I have conducted various comparative analyses using the broader family. In doing so, I have identified both subtle and considerable differences that may account for some of the unique characteristics of each family member. Here, I present a compilation of those comparative studies and identify several critical factors that may define the unique regulatory controls imparted by individual members of the Smyd Family.

5.2 Background

Smyd1, is the founding member of the Smyd family and has been most extensively studied. It is driven by two discrete promoters and encodes distinct proteins expressed in T-lymphocytes (Smyd1C) and striated muscle (Smyd1A and Smyd1B) [129]. Targeted disruption of the Smyd1 gene revealed that it is necessary for cardiomyocyte differentiation and cardiac morphogenesis [104]. More recently, several interaction partners have been observed for Smyd1B including skNAC, TRB3, MICAL, and HELZ. The skNAC protein is a

transcriptional activator specific to heart and skeletal muscle [105, 177]. TRB3, a regulator of mitosis [178], has been shown to enhance the transcriptional repression of Smyd1B that is mediated by class I HDACs. MICAL has a suspected role in cytoskeletal regulation [113] and HELZ is an RNA helicase that also interacts with RNA polymerase II [106].

Smyd3 has been noted for its involvement in the proliferation of cancer cells [106, 152]. It is over-expressed in most hepatocellular and colorectal carcinomas and its transfection into NIH3T3 cells significantly augments growth [106]. Reciprocally, knockdown of Smyd3 with siRNA has a suppressive effect on the propagation of cancer cells. Insights into potential mechanisms for Smyd3-mediated enhancement are provided by two important findings. A ternary complex forms between Smyd3 and RNA polymerase II in which HELZ serves in a bridging capacity [106]. Secondly, Smyd3 functions as a transcriptional activator by catalyzing the methylation of H3K4 [106].

In addition to the findings above, several commonalities have been established among Smyd family members. Smyd1B, Smyd2, and Smyd3 interact directly with class I HDACs and this interface facilitates their potential role in transcriptional repression. Similarly, all three interact with the nuclear receptor corepressor, N-CoR, and components of the Sin3 repression complex, both of which are known to recruit HDACs. Although the presence of EBNA2 converts Smyd1B, Smyd2, and Smyd3 into transcriptional activators, only Smyd1B and Smyd2 exhibit the ability to interact with EBNA2. Likewise, Smyd3

is excluded from the shared abilities of Smyd1B and Smyd2 to strongly interact with skNAC.

5.3 Results

5.31 Smyd Family Interactions

In an effort to broadly shed light on the shared mechanisms of the Smyd family and to rapidly search for potential Smyd-methylation targets, I searched for proteins that interact with members of the Smyd family. The delineation of Smyd-associating proteins permits valuable extrapolation of Smyd protein functions based on the known roles and pathways of their interacting partners. As numerous proteins have already been demonstrated to associate with Smyd1 and Smyd3, I focused my efforts on establishing whether these are shared interactions, among the Smyd family, or if they are unique to individual Smyd proteins.

HELZ

HELZ is an RNA helicase that was first demonstrated to associate with Smyd1B by the laboratory of our collaborator, Dr. Deepak Srivastava. It was later shown to interact with Smyd3 by Hamamoto et al. [106]. In both cases, HELZ was pulled out of a yeast two-hybrid screen and the association was

subsequently validated by co-immunoprecipitation of HELZ with the respective Smyd protein.

RNA helicases function in transcription by binding to transcription factors and RNA polymerase II (RNAPII). Based on this fact Hamamoto et al. predicted that Smyd3 may function in a transcriptional complex with RNAPII. This conjecture was confirmed by showing that Smyd3 co-immunoprecipitates with RNAPII and served as the basis for a model where HELZ acts in a bridging capacity for the association between Smyd3 and RNAP II (Fig. 4.1B) [106].

To determine if Smyd2 can also interact with HELZ, I conducted a series of co-immunoprecipitation experiments. In transient transfection experiments in 293T cells, HELZ-Flag interacted specifically with Smyd2-Myc upon immunoprecipitation with anti-Myc antibodies (Fig. 5.1A). Consistently, when cell extracts from 293T cells over-expressing Smyd2-Myc and HELZ-Flag were immunoprecipitated with anti-Flag antibodies, the immune complexes contained Smyd2-Myc. Thus, I concluded that HELZ is a shared association partner among the Smyd family of proteins.

RNA polymerase II

Based on the association between Smyd3 and RNAPII, documented in the initial characterization of Smyd3 [106], I tested the abilities of Smyd1 and Smyd2 to associate with RNAPII. In transient transfection experiments in HeLa cells,

Smyd2-Myc and Smyd3-Myc interacted specifically with endogenous RNAPII upon immunoprecipitation with anti-RNAPII antibodies (Fig. 5.1B). Smyd1B also interacted with RNAPII, though it exhibited a much lower affinity (Fig. 5.1B). Thus, I concluded that RNAPII is likely a shared association partner among the Smyd family of proteins.

MICAL

Molecule interacting with CasL (MICAL), a protein that has a suspected role in cytoskeletal regulation, was demonstrated to associate with Smyd1B by the laboratory of our collaborator, Dr. Deepak Srivastava [113]. Initially pulled out of a yeast two-hybrid screen, its association was later validated by co-immunoprecipitation of MICAL with Smyd1B. To test if it is a shared association partner among the Smyd family, I conducted a series of co-immunoprecipitations. In transient transfection experiments in 293T cells, MICAL-Flag interacted specifically with Smyd1B-Myc, but not with Smyd2-Myc or Smyd3-Myc, upon immunoprecipitation with anti-Myc antibodies (Fig. 5.1C). Likewise, when cell extracts from 293T cells over-expressing MICAL-Flag, along with either Smyd1-Myc, Smyd2-Myc, or Smyd3-Myc, were immunoprecipitated with anti-Flag antibodies, the immune complexes contained only Smyd1-Myc. Thus, I concluded that MICAL is an association partner unique to Smyd1 in the Smyd family of proteins.

Frizzled 2

Mfz10 (the murine ortholog of human Frizzled2) is a member of the Frizzled family of proteins which have a role as cell-surface receptors for the Wnt family of signal ligands. Wnt signaling pathways are involved in the regulation of cell differentiation, proliferation and embryogenesis [179-181]. Mfz10, which is highly expressed during cardiac morphogenesis [182] was identified as a protein that associates with Smyd1B in a yeast two-hybrid screen conducted by the laboratory of Dr. Deepak Srivastava. This interaction alludes to a possible function for Smyd1 in Wnt signaling.

To determine if the broader Smyd family may have a role in Wnt signaling, I conducted a series of co-immunoprecipitations involving individual Smyd proteins and Frizzled2. Surprisingly, in transient transfection experiments in 293T cells, Frizzled2-HA failed to interact with Smyd1B-Myc. It also failed to interact with Smyd3-Myc. It did, however, associate specifically with Smyd2-Myc upon immunoprecipitation with anti-Myc antibodies (Fig. 5.1D). Similarly, when cell extracts from 293T cells over-expressing Frizzled2-HA, along with either Smyd1-Myc, Smyd2-Myc, or Smyd3-Myc, were immunoprecipitated with anti-HA antibodies, the immune complexes contained only Smyd2-Myc. Personal communication with our collaborators in the Srivastiva Laboratory indicated that they too had failed to validate an association between Smyd1B and Mfz10 (Frizzled-2) via co-immunoprecipitation. Thus, I concluded that Frizzled-2 is an association partner unique to Smyd2 in the Smyd family of proteins.

Smyd family homo- and hetero-associations

Preliminary findings by Dr. Li Zhu and Dr. Robert Sims indicate that Smyd1 may self-associate and function as a homo-trimer (personal communications). Indeed, the crystal structure of Smyd3 suggests that Smyd proteins could self-associate by way of their C-terminal domain without creating a likely source of steric hindrance near their enzymatic active sites. Knowledge of homo- and/or hetero- associations among Smyd proteins would provide significant insight into the mechanistic basis of their functions. Therefore, in order to determine if the Smyd proteins are capable of forming homo- and/or hetero-Smyd complexes, I conducted a series of co-immunoprecipitation experiments using Smyd fusion proteins.

In transient transfection experiments in 293T cells, Smyd1B-GAL4 interacted specifically with Smyd1B-Myc upon immunoprecipitation with anti-Myc antibodies (Fig. 5.2A). Likewise, when cell extracts from 293T cells over-expressing Smyd1B-Myc and Smyd1B-GAL4 were immunoprecipitated with anti-GAL4 antibodies, the immune complexes contained Smyd1B-Myc. In both cases, Smyd1B-GAL4 failed to interact with a non-Smyd fusion protein (Bright-Myc). In parallel assays, Smyd2 and Smyd3 also demonstrated a propensity to self-associate (Fig. 5.2A). Thus, I concluded that Smyd1B, Smyd2, and Smyd3 each have the capacity to form homo-complexes. Interestingly, each Smyd was also capable of co-immunoprecipitating with the other Smyd proteins (Fig. 5.2A).

Therefore, I additionally concluded that the Smyd proteins are competent for forming hetero-Smyd complexes.

5.32 Smyd family methylation targets

As described in chapter 1.5, most histone lysine methylation is catalyzed by proteins containing a SET domain [27, 60]. The Smyd family comprises a subset of SET domain-containing proteins that have unique domain architecture. Specifically, this family of proteins is defined by a SET domain that is split into two segments by an MYND domain, followed by a cysteine-rich post SET domain [104]. At the onset of my research on the Smyd family, no methylation activity had been demonstrated for any of the Smyd proteins. Shortly into my tenure, Hamamoto et al. published the initial characterization of Smyd3 which demonstrated for the first time that the split SET domain of the Smyd proteins is competent for methylation [106]. Using the histone methyltransferase protocol published in the Smyd3 study, I soon confirmed the enzymatic function of Smyd3. In addition, I was also able to demonstrate the capacity of Smyd2 to dimethylate H3K36 as noted in chapter 3.33 [100]. My earliest studies also indicated that Smyd1B has a marginal capacity to methylate H3, relative to the degree of methylation catalyzed by Smyd2 (Fig. 5.3A). The enzymatic specificity of Smyd1B was later more specifically defined to be H3K4 [130]. Since then, I have demonstrated that Smyd1B can also methylate a site on H4 that has yet to be delineated (Fig. 5.3B). This finding has been independently validated by our

informal collaboration with Dr. Yali Dou at the University of Michigan. Most recently, Dr. Li Zhu discovered the ability of Smyd1A and Smyd1B to methylate the muscle-specific transcription factor, skNAC (personal communication) and he is currently investigating the biological implications of this event.

skNAC

skNAC, is a muscle-specific isoform of the ubiquitously expressed αNAC that acts as a transcriptional activator of myoglobin [177]. It has been shown to interact with both Smyd1A and Smyd1B [105]. For this interaction to occur, both the MYND and the N-SET domains of Smyd1 are necessary. A PXLXP motif in skNAC is also required [105]. Smyd1 expression overlaps with that of skNAC during cardiac development when Smyd1-mediated methylation of skNAC is believed to regulate muscle-specific gene expression. Interestingly, although Smyd1A and Smyd1B have both been shown to methylate skNAC *in vitro*, only Smyd1B has the ability to methylate this substrate *in vivo*.

I have shown that both Smyd2 and Smyd3 are expressed in the heart (Fig. 3.1B). As Smyd2 has also been noted for its ability to associate with skNAC in co-immunoprecipitations (Dr. Robert Sims; personal communication), it is reasonable to consider that skNAC may be a common methylation target among Smyd family members. To determine if Smyd2 and Smyd3 can methylate skNAC, I conducted a series of methyltransferase assays using a fragment of skNAC termed E34 (5.3A). E34 encodes amino acids 1857-2187 of skNAC and

was originally identified in a Smyd1B yeast two-hybrid screen [105]. E34 methylation was tested after incubation of Smyd2 or Smyd3 with S-adenosyl-L-[methyl-^{3}H] methionine (SAM) and recombinant E34, as a substrate. A band corresponding to ^{3}H-labelled E34 was seen with both Smyd2 and Smyd3 in the fluorogram (Fig. 5.5), indicating that skNAC may be a common methylation target of the Smyd family. To delineate the region of E34 that is methylated by Smyd3, I repeated the methyltransferase assay using a truncated fragment of skNAC. Although the positive control, Smyd1B, robustly methylated this fragment, there was no methylation in the presence of Smyd3 (Fig. 5.5). Therefore, I concluded that although skNAC is likely a common methylation target of the Smyd family, the Smyd proteins act on distinct residues within this substrate.

FKBP38

FKBP38 is a cytophilin involved in Hedgehog- and calcineurin/NFAT-mediated signaling. It has also been implicated in Bcl2-mediated apoptosis [112, 183]. It was identified as a potential Smyd1-associating protein in a yeast two-hybrid analysis conducted by Dr. Robert Sims (personal communication) and this was later validated by co-immunoprecipitation experiments. More recently, Hui Nie (Tucker Laboratory) demonstrated the co-localization of Smyd1C with FKBP38, *in vivo*. She predicted that this interaction inhibits Bcl2-mediated apoptosis based on phenotypes that she observed associated with the

conditional deletion of Smyd1 in mice. She further proposed that this inhibition may be dependent upon the methylation of FKBP38 by Smyd1C.

To determine if FKBP38 is a potential methylation target of Smyd proteins, FKBP38 methylation was tested after incubation of Smyd1B, Smyd1C, Smyd2, or Smyd3 with S-adenosyl-L-[methyl-^{3}H] methionine (SAM) and recombinant FKBP38, as a substrate. A separate reaction including Smyd1B, SAM, and E34 was conducted as a positive control. A band corresponding to ^{3}H-labelled E34 was observed with Smyd1B in the fluorogram (Fig. 5.6A), indicating that the reaction conditions were adequate for lysine methylation. However, no methylation was observed in any of the lanes containing FKBP38 as the substrate (Fig. 5.6A. Therefore, I concluded that FKBP38 is not likely to be a methylation target of Smyd1B, Smyd1C, Smyd2, or Smyd3.

MICAL

As mentioned in chapter 5.31, MICAL, a protein that has a suspected role in cytoskeletal regulation, was demonstrated to associate with Smyd1B by the laboratory of our collaborator, Dr. Deepak Srivastava [113]. Initially pulled out of a yeast two-hybrid screen, its association was later validated by co-immunoprecipitation of MICAL with Smyd1B. Recently, Hui Nie demonstrated that MICAL also co-immunoprecipitates with Smyd1C (personal communication) and she proposed that MICAL may be a methylation substrate of Smyd1.

To determine if MICAL is a potential methylation target of Smyd proteins, MICAL methylation was tested after incubation of Smyd1B, Smyd1C, Smyd2, or Smyd3 with SAM and recombinant MICAL, as a substrate. A separate reaction including Smyd1B, SAM, and E34 was conducted as a positive control. A band corresponding to ^{3}H-labelled E34 was observed with Smyd1B in the fluorogram (Fig. 5.6A), indicating that the reaction conditions were adequate for lysine methylation. However, no methylation was observed in any of the lanes containing MICAL as the substrate (Fig. 5.6A). Therefore, I concluded that MICAL is not likely to be a methylation target of Smyd1B, Smyd1C, Smyd2, or Smyd3.

RNA polymerase II

I have demonstrated that Smyd1B, Smyd2, and Smyd3 associate with RNAPII in HeLa cells (chapter 5.31). To determine if RNAPII is a potential methylation target of Smyd proteins, methylation of the C-terminal domain of RNAPII (RNAPII-CTD) was tested after incubation of Smyd1B, Smyd2, or Smyd3 with SAM and recombinant RNAPII-CTD, as a substrate. A separate reaction including Smyd2, SAM, and histone H3 was conducted as a positive control. A band corresponding to ^{3}H-labelled H3 was observed with Smyd2 in the fluorogram (Fig. 5.6B), indicating that the reaction conditions were adequate for lysine methylation. However, no methylation was observed in any of the lanes containing RNAPII-CTD as the substrate (Fig. 5.6B). Therefore, I concluded that

RNAPII-CTD is not likely to be a methylation target of Smyd1B, Smyd1C, Smyd2, or Smyd3.

5.34 Effects of Smyd family members on cell proliferation

The role of Smyd3 in transcriptional regulation as a histone methyltransferase has been linked to its ability to augment cellular proliferation [106]. To investigate the relative effects that Smyd1 and Smyd2 may have on cell proliferation, NIH3T3 cells were transfected with either Smyd1-Myc, Smyd2-Myc, or Smyd3-Myc. Relative to the mock control and consistent with previous findings [106], over-expression of Smyd3 markedly increased cell growth (Fig. 5.7). Conversely, the transfection of 3T3 cells with Smyd1 or Smyd2 led to a decrease in their proliferation (Fig. 5.7), indicating potential roles for Smyd1 and Smyd2 in the maintenance of cell-cycle progression.

5.4 Discussion

The SMYD Family is a group transcriptional regulators that function, in part, through histone modifications. Despite their similar architecture and enzymatic mechanisms, Smyd proteins induce dramatically disparate outcomes on cell proliferation. Here, I have identified both subtle and considerable differences that may account for some of the unique characteristics of each family member. I present this data as a comparative study that highlights

unique factors which may help to define the unique regulatory controls imparted by individual members of the Smyd Family.

5.41 Smyd family protein interactions

Smyd role in RNAPII transcription complexes

A growing theme in our characterization of the Smyd family is that the functions of individual Smyd proteins may vary greatly, according to their associations with distinct complexes. Here, I have identified two proteins that represent a shared association among Smyd family members. These are the RNA helicase, HELZ, and RNA polymerase II. As these two proteins are known to function together in transcriptional complexes, these associations link the methyltransferase activity of each Smyd protein with transcriptional regulation. This is an important finding, because there is currently much debate regarding how histone modifications are targeted to specific sites. These findings strengthen the notion that HKMTs are recruited to histones through their interactions with DNA-binding proteins and other constituents of transcription complexes.

There are several common forms of RNAPII involved with different stages of transcription [184]. These forms vary according the the phosphorylation state of their CTD. When RNAPII is hypophosphorylated at its CTD, it is known to

interact specifically with components of the transcriptional pre-initiation complex [184]. Phosphorylation at RNAPII-CTD serine 5 is associated with transcriptional initiation and early elongation complexes [185]. Finally, RNAPII phosphorylated at serine 2 of its CTD is associated with progressive transcriptional elongation [185]. It will be informative, therefore, to determine if the Smyd proteins associate with a specific form of RNAPII.

Smyd self-associations

My findings indicating that Smyd proteins can both self associate and/or interact with one another gives rise to many potentially convoluted pathways that will undoubtedly be daunting to sort out. In the context of this thesis, I will touch on just one relatively simple paradigm involving the interaction of Smyd proteins at transcriptional complexes. As the Smyd family is shown here to interact with RNAPII, I will relate the potential for Smyd proteins to associate in common complexes with a model illustrating their prospective involvement with different forms of the RNAPII transcription machinery.

In *S. cerevisiae*, the methyltransferases, SET1 and SET2, have been shown to interact with the initiation/early elongation and processive forms of the RNAPII transcription machinery, respectively. SET1, which associates specifically with the serine 5-phosphorylated RNAPII complex, trimethylates H3K4, thereby opening the structure of chromatin and facilitating the recruitment of processivity factors and the 5' mRNA capping machinery [186-188]. SET2,

which associates specifically with the serine 2-phosphorylated RNAPII complex, follows and dimethylates H3K36 along the length of the transcribed gene [186, 188]. Dimethylated H3K36 is associated with the recruitment of the HDAC, Rpd3, in *S. cerevisiae* [127]. As histone deacetylation corresponds with transcriptionally inaccessible heterochromatin, a model has been proposed where SET1 and SET2 cooperate to achieve a rapid but short-lived transcriptional response [127, 188]. In *S. cerevisiae*, this may be linked to critical survival mechanisms, such as the toxic stress response, where limited expression of specific genes is required [189].

In a mammalian model, an analogous rapid transcription response mechanism may be ascribed to the Smyd family. Specifically, the H3K4 activity of Smyd1 or Smyd3 may be associated with the serine 2-phosphorylated RNAPII complex, initiating transcription of specific genes. Smyd2, which has been shown to associate with the human ortholog of Rpd3, HDAC1 (chapter 3.34) [100], could then follow, by interacting with the serine 5-phosphorylated RNAPII elongation complex, and methylate H3K36. Finally, histone deacetylation by the associating HDAC1 would then silence transcription of the proximal genes.

5.42 skNAC methylation by the Smyd family

In my screen of potential Smyd methylation targets, I identified skNAC as a common Smyd family substrate. In preliminary assays, it appears that the site of skNAC methylation by individual Smyd proteins may be distinct. Specifically,

Smyd1 methylates a site within the unique region of skNAC while Smyd3 targets a site in skNAC that is shared with the C-terminal residues of its splice variant, αNAC. The αNAC protein is a transcriptional co-activator that is known to intereract with the c-Jun transcription factor [177, 190]. These findings suggest that in addition to skNAC, αNAC should be investigated for its capacity to be methylated by Smyd proteins. As Smyd1 expression is restricted to heart and skeletal muscle, its methylation of a site that is in the unique region of skNAC is consistent with its expression pattern. Similarly, since αNAC is broadly expressed in various tissues, its methylation by Smyd2 and/or Smyd3 would be in keeping with the broad expression of those Smyd enzymes.

5.43 Smyd family role in methyltransferase-mediated regulation of p53

Smyd2 was recently demonstrated to methylate K370 of the tumor suppressor, p53, and this modification was shown to repress p53-mediated transcription [172]. Although Smyd2 has only the capacity to monomethylate p53, it was determined that p53 is sometimes dimethylated at K370. As the C-terminal region of p53 contains the consensus Smyd interacting motif, PXLXP, it is reasonable to speculate that another Smyd family member may be responsible for the dimethylation of p53. Therefore, we are currently in the process of screening the potential of other Smyd family members to methylate p53.

5.5 Conclusion

I have identified both subtle and considerable differences that may account for some of the unique characteristics of each Smyd family member. Although they all interact with the RNAPII transcription machinery, it is likely that they associate with distinct forms of that complex. Likewise, while they are each capable of methylating the muscle-specific transcription factor, skNAC, it appears that individual Smyd proteins methylate distinct residues on skNAC. As has been shown with other transcription factors, such as p53, modest differences in points of methylation can produce drastically disparate outcomes on transcription [172]. The Smyd family members are also shown to have distinct effects on cell proliferation. These studies indicate that future research on Smyd proteins, with strong emphasis on the unique organismal context, will shed light onto the biological functions of Smyd family proteins. Such emphasis may reveal new insights into the relationships between chromatin modifications and the development and differentiation of tissues and organisms.

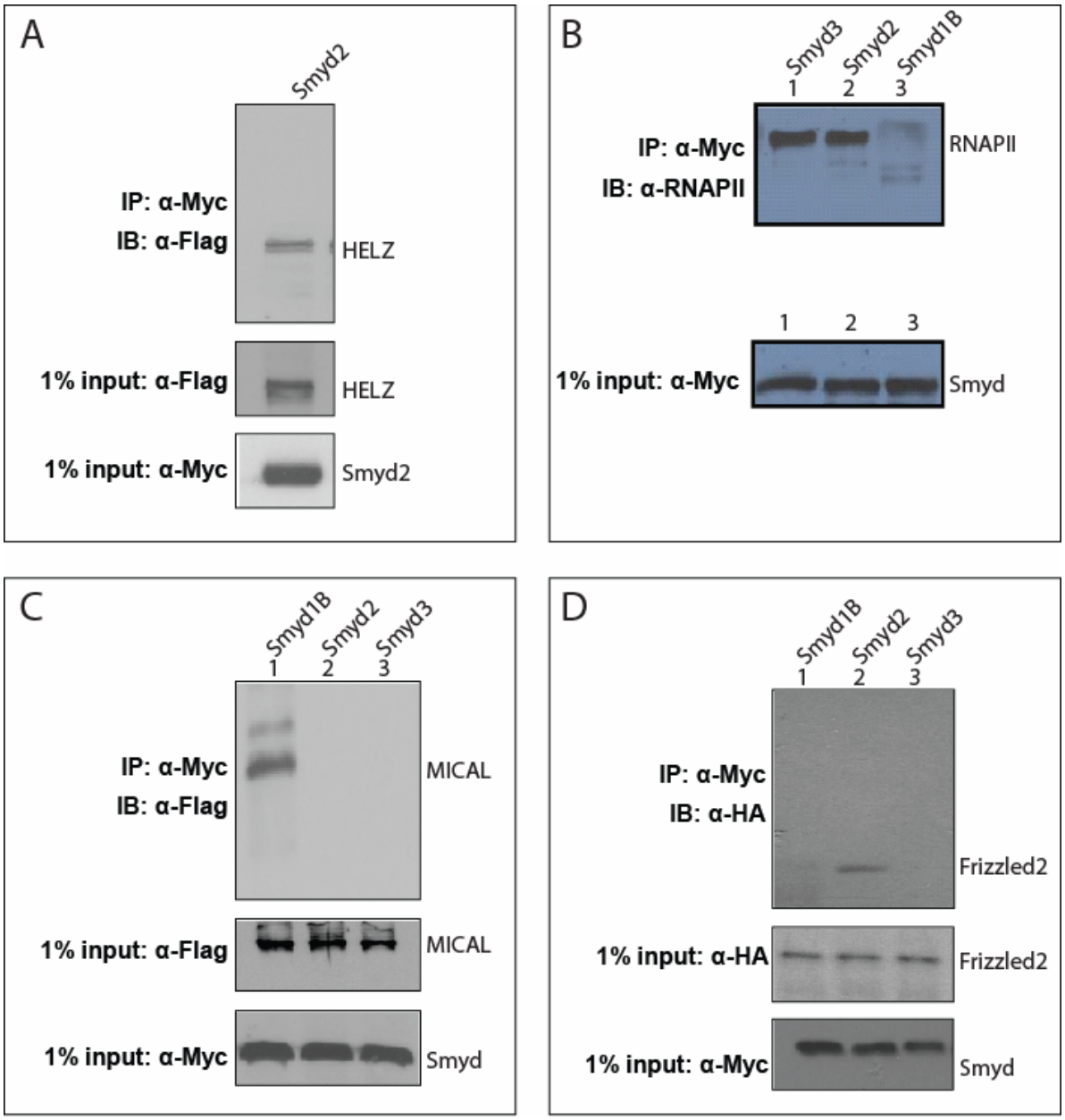

Figure 5.1: Characterization of Smyd family protein interactions. (A) Smyd2 associates with HELZ. HeLa cells were co-transfected with Smyd2-Myc and HELZ-Flag and, 48 hours post-transfection, whole RIPA lysates were prepared. Antibodies directed against Myc were used for immunoprecipitation, followed by western analysis using antibodies directed against Flag (upper panel). Input is shown in lower panels. (B) Smyd proteins associate with RNAPII. HeLa cells were transfected with either Smyd1B-Myc, Smyd2-Myc, or Smyd3-Myc and lysates were prepared as detailed above. Antibodies directed against Myc were

used for immunoprecipitation, followed by western analysis using antibodies directed against RNAPII (upper panel). Input is shown in lower panel. (C) Smyd1B interacts with MICAL. 293T cells were co-transfected with MICAL and either Smyd1B-Myc, Smyd2-Myc, or Smyd3 Myc and lysates were preparared as detailed above. Antibodies directed against Myc were used for immunoprecipitation, followed by western analysis using antibodies directed against Flag (upper panel). Input is shown in lower panel. (D) Smyd2 interacts with Frizzled2. 293T cells were co-transfected with Frizzled2-HA and either Smyd1B-Myc, Smyd2-Myc, or Smyd3-Myc and lysates were preparared as detailed above. Antibodies directed against Myc were used for immunoprecipitation, followed by western analysis using antibodies directed against the HA epitope (upper panel). Input is shown in lower panel.

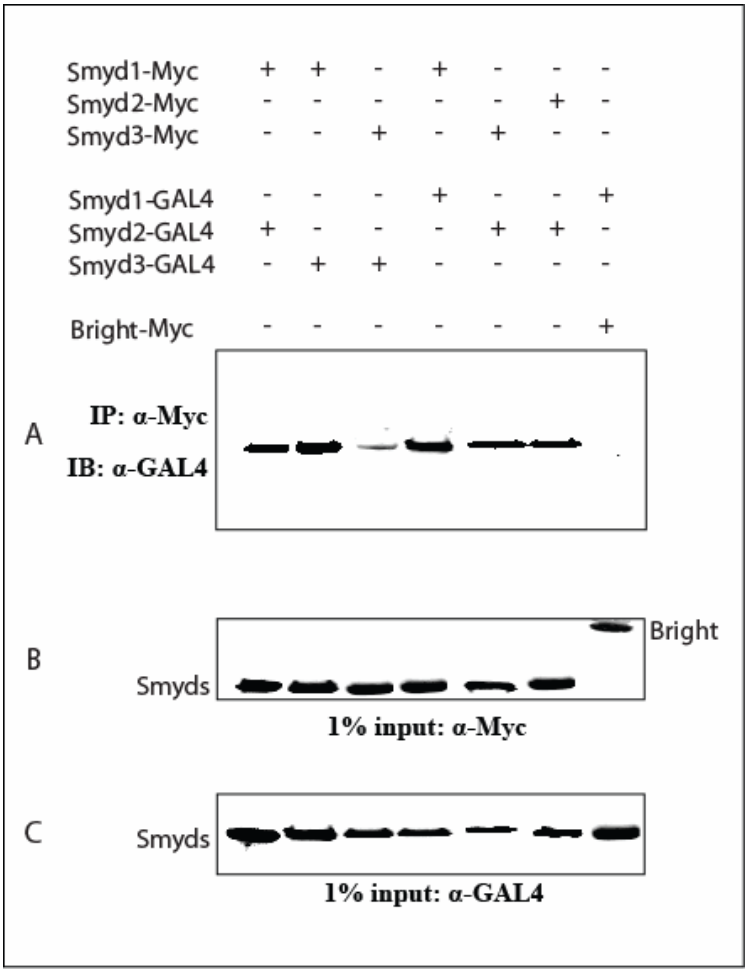

Figure 5.2: Smyd family members self-associate. (A) Smyd family members form homo- and hetero-complexes. 293T cells were co-transfected with Smyd-Myc and Smyd-GAL4 fusion proteins as indicated and, 48 hours post-transfection, whole RIPA lysates were prepared. Antibodies directed against Myc were used for immunoprecipitation, followed by western analysis using antibodies directed against the GAL4 epitope. Bright-Myc, which does not associate with Smyd proteins, was included as a negative control. (B) Smyd-Myc and Bright-Myc protein inputs are shown. (C) Smyd-GAL4 protein inputs are shown.

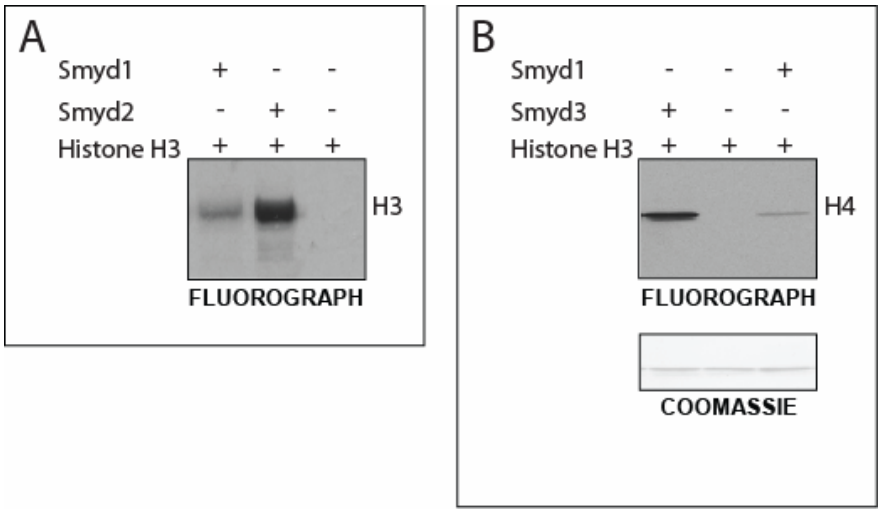

Figure 5.3: Smyd1B has a marginal capacity to methylate histones H3 and H4, relative to Smyd2 and Smyd3, respectively. (A) Smyd1B and Smyd2 methylate histone H3 in an *in vitro* histone methyl transferase (HKMT) assay using 1µg recombinant H3 as substrate. The fluorograph shows a 17kD band, corresponding with histone H3, as indicated. (B) Smyd1B and Smyd3 methylate histone H4 in an *in vitro* HKMT assay using 1µg recombinant H4 as substrate. Fluorograph is shown in the upper panel; the ~11 kD band, corresponding with Histone H4, is indicated; a coomassie stained membrane was used to verify equal loading and is depicted in the lower panel.

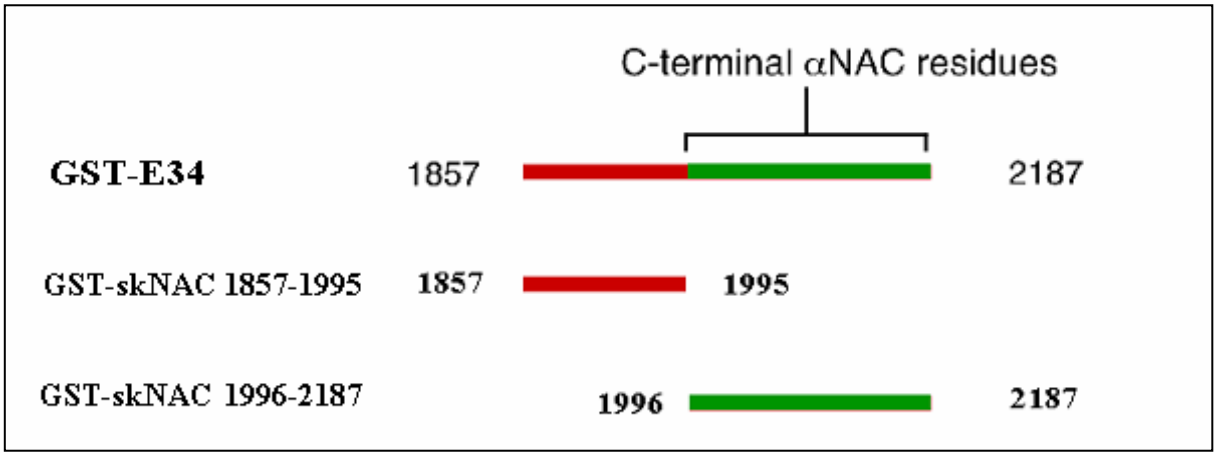

Figure 5.4: E34 fragments of skNAC. E34 encodes amino acids 1857-2187 of skNAC and was originally identified in a Smyd1B yeast two-hybrid screen [105]. Shown here is full-length E34 (top), including a region that is shared with the C-terminal residues of the skNAC splice variant, αNAC (green) and a region that is unique to skNAC (red). Two truncations of E34 have been constructed, including an amino-terminal fragment (middle) that contains the region of E34 encompassing skNAC residues 1857-1995, which are unique to skNAC, and a C-terminal fragment (bottom) that contains the region encompassing skNAC residues 1996-2187, which are shared with αNAC. (Adapted from Zhu 'committee meeting')

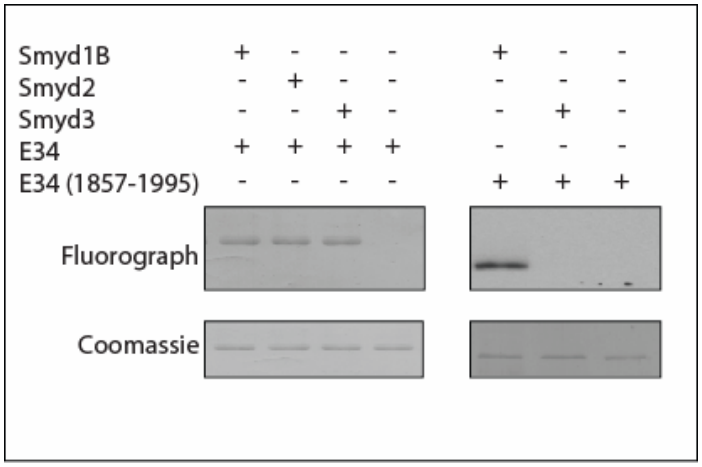

Figure 5.5 Methylation of the E34 fragment of skNAC by members of the Smyd family. Smyd1B, Smyd2, and Smyd3 methylate a fragment of skNAC in an *in vitro* methyltransferase assay using 1µg recombinant E34 as substrate (upper left). A coomassie stained membrane was used to verify equal loading of E34 (lower left). Smyd1B, but not Smyd3, methylates an E34 truncation (1857-1995) that contains only the residues of E34 which are unique to skNAC (upper right). A coomassie stained membrane was used to verify equal loading of the E34 truncation (lower right).

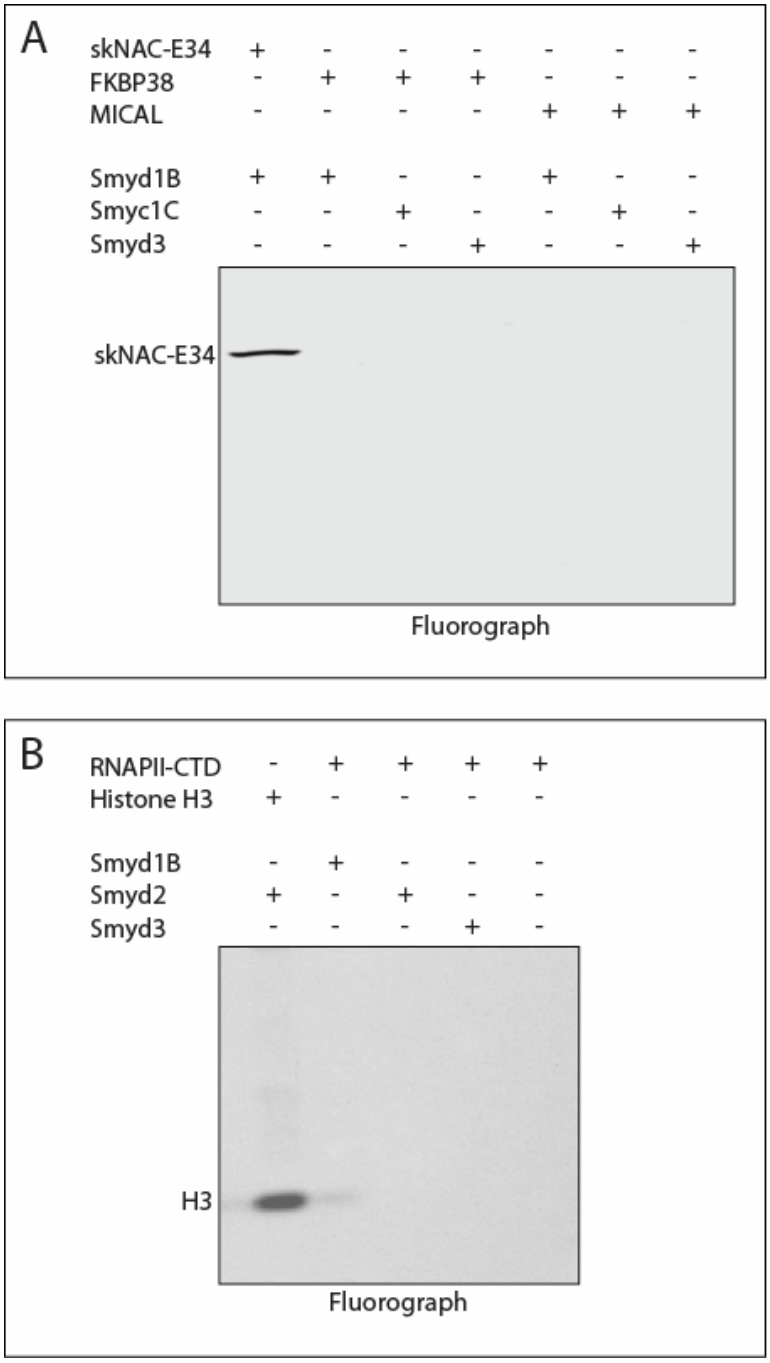

Figure 5.6: Characterization of Smyd family methylation substrates. (A) Smyd1B, Smyd1C, and Smyd3 fail to methylate FKBP38 or MICAL in an *in vitro* methyltransferase assay. A separate reaction including Smyd1B as enzyme and E34 as substrate was conducted as a positive control. A band corresponding to ^{3}H-labelled E34 is shown in the fluorograph, indicating that the reaction

conditions were adequate for lysine methylation. (B) Smyd1B, Smyd2, and Smyd3 fail to methylate the C-terminal domain of RNA polymerase II (RNAPII-CTD) in an *in vitro* methyltransferase assay. A separate reaction including Smyd2 as enzyme and histone H3, as substrate, was conducted as a positive control. A band corresponding to ^{3}H-labelled H3 is shown in the fluorograph, indicating that the reaction conditions were adequate for lysine methylation.

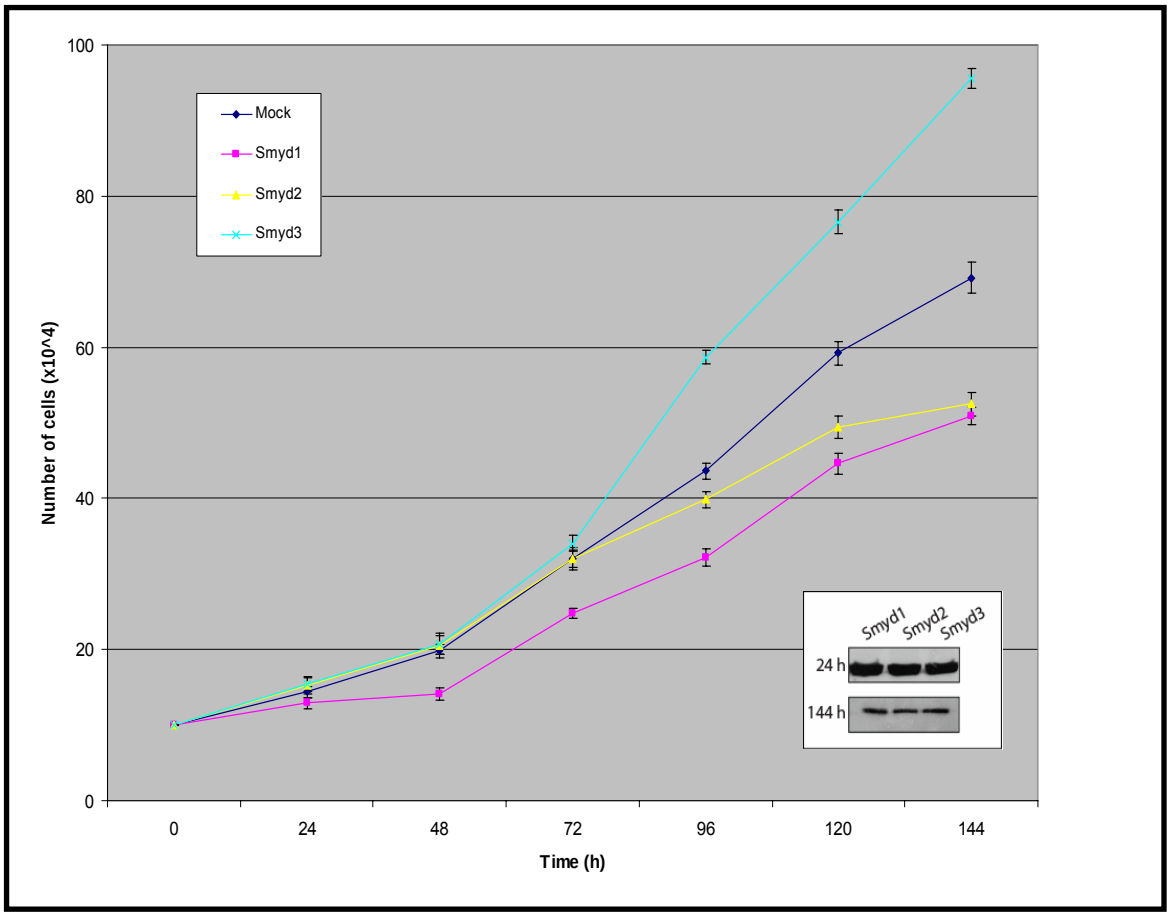

Figure 5.7: Effects of Smyd family members on cell proliferation. NIH3T3 cells were transfected with plasmids encoding Myc-tagged Smyd1B, Smyd2, or Smyd3. Cells transfected with the empty expression construct (Mock) served as control. All cells were monitored by cell counting using trypan blue exclusion. The inserts show the level of expression of Smyd1-Myc Smyd2-Myc and Smyd3-Myc at 24 and 144 hours post-transfection, demonstrating similar levels of ectopically introduced proteins in the NIH3T3 cells. Whereas ectopically introduced Smyd3 enhanced the proliferation, Smyd1 and Smyd2 displayed a negative effect on the growth rate of NIH3T3 cells.

6. THE ROLE OF HSP90 IN SMYD FAMILY EXPRESSION AND CATALYSIS

6.1 Abstract

The molecular chaperone, Hsp90, facilitates the maturation and/or activation of over 100 'client proteins' involved in cell signal transduction and transcriptional regulation. Largely an enigma among the families of heat shock proteins, it is central to processes broadly ranging from cell cycle regulation to cellular transformation. Hsp90 was recently shown to interact with Smyd3, a methyltransferase involved in colorectal, hepatocellular, and breast cell carcinogenesis, and this association was demonstrated to enhance the catalytic activity of Smyd3. Here I investigate the potential for Hsp90 to function broadly in the enhancement of SET-containing methyltransferases. In addition, as Hsp90 has long been noted for its capacity to impede the *in vitro* aggregation of a broad range of non-specific proteins induced to express in *E. coli*, I analyze the ability of Hsp90 to improve the otherwise marginal bacterial expression of the Smyd family of proteins.

6.2 Background

Hsp90 defines a family of molecular chaperones that are highly conserved from prokaryotes to eukaryotes [191-195]. Nonessential for normal growth in most bacteria, Hsp90 is abundantly expressed in higher eukaryotes where it has been shown to be necessary for viability [196, 197]. It functions as a homodimer

that associates with co-chaperones to catalyze the maturation and/or activation of over 100 substrate proteins that are known to be involved in cell regulatory pathways [195]. These 'client proteins' include protein kinases, nuclear hormone receptors, transcription factors, and an array of other essential proteins [198]. While much is known regarding the ATPase-driven conformational cycling of Hsp90, the precise physical effects imparted by this chaperone that serve to activate its substrates are still poorly understood [195].

Hsp90 architecture

Three highly conserved domains comprise the structure of Hsp90. These include the N-terminal domain, responsible for ATP-binding, a proteolytically resistant core domain, and the C-terminal domain that facilitates homodimerization (Fig. 6.1A) [199, 200]. In eukaryotes, a more variable charged region links the N-terminal domain to the core domain. The length and composition of this linker region is highly divergent among organisms [201]. As no atomic resolution structure for full-length Hsp90 is yet available, the most thorough structural analyses for Hsp90, to date, have been based on crystallographic studies of its individual domains.

A mechanistic understanding of Hsp90 was nebulous until partial sequence homology was recognized between its N-terminal domain and two types of ATP-dependent proteins. These included the type II topoisomerases and the MutL DNA mismatch repair enzymes [202]. Structural analyses of the N-

terminal domain of Hsp90 revealed that this domain contains an ATP-binding site [203]. Additionally, biochemical studies suggest that transitory interactions between two N-terminal domains of the Hsp90 homodimer occur in an ATP-dependent manner, and this provides the mechanistic basis for an ATPase-driven molecular clamp [204]. Mutations in this region that impair the ability of Hsp90 to either bind or hydrolyze ATP eliminate its chaperone activity [205]. The discovery that the antibiotics, radicicol and geldanamycin, inhibit the Hsp90-dependent activation of numerous regulatory and signal transduction proteins by occupying this ATP-binding site was a revelation in antitumor research [206]. Indeed, this is the origin of almost a decade of intense efforts focusing on Hsp90 as a therapeutic target for the treatment of cancer.

Biochemical and structural analyses of the core domain of Hsp90 determined that this domain contains a catalytic loop that accepts the γ-phosphate of ATP [207]. This led to the characterization of Hsp90 as a “split ATPase” [207]. Structural and mechanistic similarities shared between Hsp90 and DNA gyrase B serve as the basis for suggesting that the core domain is also involved in the interface of Hsp90 with its client proteins. Strengthening this position, several studies now implicate the core of Hsp90 in its interactions with p53, eNOS, and Akt [208-210].

The advent of the C-terminal crystal structure provided further evidence for the antiparallel dimeric architecture of Hsp90 that had been previously predicted by electron microscopy [211-213]. C-terminal truncations of Hsp90 abolish its ability to hydrolyze ATP, indicating that its dimeric nature is essential

for its activity [204]. A highly conserved pentapeptide (MEEVD) present in the C-terminus of eukaryotic Hsp90 is recognized by co-chaperones containing tetratricopeptide repeats [214-216]. Thus, the C-terminal domain is also involved in the formation of active Hsp90 multiprotein complexes.

The structural mechanism for the chaperone activity of Hsp90 has been likened to a 'molecular clamp.' In the absence of bound nucleotide, the N-termini of the Hsp90 homodimer maintain an open-state, facilitating the 'capture' of client proteins (Fig. 6.1B) [204]. Association with ATP induces modest changes to the conformation of Hsp90 that permit a transitory interaction between the opposing N-terminal domains. This produces the closed-form of Hsp90 where clamping of the substrate protein occurs [204]. It is through this ATPase-driven cycle that Hsp90, with the assistance of several co-chaperones, induces the activation of its 'clientelle' [217].

Hsp90 co-chaperones

Hsp90 is not capable of autonomously functioning as a protein chaperone. Instead, it serves at the core of various multiprotein complexes that incorporate other chaperones, such as Hsp70, and an assortment of co-chaperones [195, 218]. The broadest class of Hsp90 co-chaperones are those containing one or more tetratricopeptide repeat (TPR) motifs that interact with the C-terminal domain of Hsp90 [219]. Beyond the conservation of their TPR motifs, these proteins are remarkably diverse, possessing few overlapping biochemical

characteristics [219]. Hop/Sti1, for example, facilitates the interaction between Hsp70 and Hsp90 [220], while WISp39 serves as a client protein specificity factor [221]. A number of TPR-containing co-chaperones even convey their own catalytic activities [195]. These include such enzymes as the E3/E4-ubiquitin ligase, CHIP [222], the protein phosphatase, PP5 [223], and several prolyl isomerases [224, 225]. It is known that CHIP functions in the targeting of Hsp90 client proteins for proteasome-mediated degradation [222]. However, the biological functions associated with the recruitment of other enzymes to Hsp90 chaperone complexes are still unclear.

Several Hsp90 co-chaperones have been shown to regulate the ATPase-driven molecular clamp cycle associated with its N-terminal domain. While Hop/Sti1, p23, and Cdc37 impair the progression of this cycle [215, 226, 227], Aha1 and Cpr6 function to enhance it [226, 228]. Because Hop/Sti1 and Cdc37 are both involved with the recruitment of Hsp90 client proteins, their inhibition of the ATPase cycle is thought to permit the loading of client proteins by maintaining the open clamp conformation of Hsp90 [227, 229]. Cpr6 is known to subsequently displace Hop/Sti1 by competing for the C-terminal TPR-recognition motif of Hsp90, thereby permitting progression of the clamp cycle [215]. The Hsp90 activation potential of Aha1 is achieved through extensive associations along the core domain of Hsp90 that induce conformational changes within the Hsp90 catalytic loop. These adjustments place the active site of the loop in better proximity for the acceptance of the γ-phosphate of ATP [230].

Hsp90 client proteins

The most detailed understanding of the effects of Hsp90 on its client proteins has been gleaned from its involvement with the maturation of steroid hormone receptors. Steroid receptors must be maintained in a labile conformation that allows them to be rapidly activated in the presence of their cognate ligand [231]. Hop1/Sti1, by virtue of its ability to bind Hsp70 and Hsp90 in tandem, facilitates the transfer of Hsp70-bound receptors to the open form of Hsp90. The Hsp90 system then induces subtle alterations in the conformation of the bound steroid receptor that enhances its affinity toward its respective ligand [232].

Protein kinases comprise the most prevalent group of Hsp90 client proteins. The co-chaperone Cdc37 is known to interact concurrently with protein kinases and Hsp90, thereby delivering client kinases to the Hsp90 chaperone complex [233, 234]. Bound to Hsp90, the client kinases are stabilized, and remain in a receptive but inactive state while awaiting appropriate signals [233]. The details of the Hsp90-protein kinase chaperone system are still being characterized.

Beyond its specific *in vivo* role in chaperoning authentic client proteins, Hsp90 has long been noted for its capacity to impede the *in vitro* aggregation of a broad range of non-specific proteins induced to express in *E. coli* [235]. Figure 6.2 shows examples of proteins for which we have used strains of E. coli that over-express Hsp90 (Plus90α^{TM}; Plus90β^{TM}; Expression Technologies Inc., San

Diego, CA) to prevent aggregation during expression [100, 106, 170, 177, 236-239]. This illustrates the structural and functional disparity among the *in vitro* clientele of Hsp90. While the selectivity of Hsp90 for its *in vivo* client proteins points to a highly specific mechanism, the architectural variation among its substrates, both *in vivo* and *in vitro*, implicate a far more general mechanism. In this way, Hsp90 remains an enigma among heat shock proteins.

Hsp90 was recently shown to interact with Smyd3, a methyltransferase involved in colorectal, hepatocellular, and breast cell carcinogenesis, and this association was demonstrated to enhance the catalytic activity of Smyd3 [106]. This led me to question whether Hsp90 may function more broadly in the enhancement of other SET-containing methyltransferases. As Smyd3 expression in *E. coli* leads to the aggregation of Smyd3 in insoluble inclusion bodies, I further speculated whether the co-expression of Hsp90 with Smyd3 may prevent such unwanted aggregation.

6.3 Results

6.31 Protein expression systems

Given the heightened availability of sequences for genomes from many organisms, protein expression is increasingly important for structural and functional analyses of proteins. Among currently available recombinant systems for protein expression, *E. coli* is used most frequently because as a host, it is

relatively inexpensive, genetically well known, and can be rapidly cultivated at high densities [240]. However, there are countless examples of less than successful expression of proteins in bacterial hosts. High level expression of proteins in *E. coli* often leads to the formation of insoluble protein aggregates, referred to as inclusion bodies [241]. Initial purification of inclusion bodies is relatively simple. However, the agglomerated proteins must be recovered using strong denaturants; in turn, the correct three-dimensional conformation must be restored by procedures which are often time-consuming, costly, and laborious. Finally, obtaining the correctly folded and active enzyme is not guaranteed. Consequently, much effort is directed to maximize the expression of proteins in soluble form.

Although optimization of expression conditions can often reduce solubility problems, this is not always sufficient. Currently, expression of proteins as fusions with affinity tags is the method of choice to achieve adequate levels of soluble protein [241] when the manipulation of growth conditions is not adequate. However, even fusion proteins can be relegated to inclusion bodies, prompting the more recent advent of methods that depend upon the co-expression of recombinant proteins with molecular chaperones [199].

6.32 The role of Hsp90 in Smyd family expression and catalysis

Among the passel of well-described and characterized chaperones, Hsp90 displays an eclectic selectivity for the type of "client" proteins it escorts [195,

242]. Currently, many research groups focus their efforts on defining how the Hsp90 chaperone machinery assists in the stabilization, assembly, or activation of client proteins. At present, Hsp90 is ascribed to promote subtle changes in conformation and/or domain arrangement in its client proteins, facilitating subsequent interactions with other molecules [195, 199, 232].

A far less subtle influence of Hsp90 has been observed on members of the Smyd protein family, reported to be involved in cell cycle regulation and the biology of neoplasms [106, 236, 243]. Whereas the SET domain of Smyd3 has H3K4 methyltransferase (HKMT) activity, which is enhanced in the presence of the heat-shock protein Hsp90α [106], the SET domain of Smyd2 mediates H3K36-specific methylation [243]. The influence of chaperones such as Hsp90 on the specificity and activity of HKMTs such as Smyd3 requires further clarification.

When expressed in a standard *E. coli* system, only marginal levels of Smyd3 or Smyd2 protein can be found in the soluble fraction (Fig. 6.2A). As expected, both of these proteins, when concentrated from the soluble fraction display HKMT activity (Fig. 6.2B). However, when isolated from the insoluble fraction and refolded, these Smyd proteins display almost no HKMT activity (Fig. 6.2B).

Failure to observe the HKMT activity of Smyd3 or Smyd2, isolated from the insoluble fractions, prompted me to seek a method for enhanced expression of these proteins in the soluble fraction. Although Hsp90α was shown to co-

immunoprecipitate with Smyd3 in HeLa cell lysates [106], Hsp90α has also been shown to form hetero-oligomers with another Hsp90 isoform, Hsp90β [244-246]. To determine unequivocally which Hsp90 isoform functions to chaperone Smyd3, I obtained *E. coli* strains expressing, separately, either the Hsp90α or the Hsp90β isoform (Plus90α™ strain and Plus90β™ strain; Expression Technologies, Inc., San Diego, CA) and expressed Smyd3 in each. Contrary to my expectation, both isoforms, Hsp90α and Hsp90β, increased the solubility of Smyd3, indicating that both isoforms may function as chaperones for Smyd3 (Fig. 6.2A). To determine if these strains could also enhance the soluble expression of Smyd2, it was also expressed in both Hsp90 strains. Similar to Smyd3, Smyd2 expression was significantly enhanced in both Hsp90 strains, relative to expression achieved in a standard BL21 strain (Fig. 6.2A).

As discussed above, Hsp90α was reported to increase the HKMT activity of Smyd3 [106]. My data indicating that both Hsp90 isoforms enhance the solubility of Smyd3 prompted me to question whether Hsp90β could also enhance the enzymatic activity of Smyd3. For these assays, Smyd3 was expressed in a standard BL21 *E. coli* strain and purified followed by a HKMT assay in the presence of recombinant HSP90α or Hsp90β. Smyd3 displayed notably higher HKMT activity in the presence of either Hsp90α or Hsp90β (Fig. 6.2C). My results demonstrate that the presence of either Hsp90 isoform is sufficient to enhance the HKMT activity of Smyd3.

Although both Hsp90 isoforms augment the expression of soluble Smyd2, neither displayed any marked effects on the HKMT activity of Smyd2 (Fig. 6.2C). This suggested that Hsp90 might assist protein solubility in a more generalized fashion. To further define the degree of specificity toward client proteins manifest by the Hsp90Plus^{TM} strains, two unrelated proteins, skNAC E34 [105] and RNA polymerase II carboxy terminal domain (RNA pol II CTD) [247] were individually expressed in each of the Hsp90 strains. Both of these proteins exhibited significantly higher soluble expression in the Hsp90Plus^{TM} strains relative to the standard BL21 strain (Fig. 6.2D).

6.4 Discussion

These data, along with the fact that Hsp90 is known to interact with over 100 different proteins [195], indicate that the Hsp90-expressing strains may be effective for enhancing the expression of many distinct proteins and therefore, dramatically broadens the potential utility of this *E. coli* expression system. I conclude that the Hsp90Plus^{TM} system offers an efficient and potentially broad-reaching approach for improving the expression of soluble and active enzymes for research and/or therapeutic purposes. The increased yield of soluble enzyme using Hsp90 strains of *E. coli* provides a simple alternative to the painstaking task of isolating inclusion bodies and attempting to refold proteins into their catalytically active form. In addition, these studies have provided new insights into the effects of Hsp90 on the catalytic activity of two histone

methyltransferases. Taken together, these data should facilitate current efforts by other laboratories toward defining how the Hsp90 chaperone machinery assists in the stabilization, assembly, or activation of client proteins.

Chaperoning tumorigenesis

The essential roles that Hsp90 fulfills in the normal physiology of healthy cells are even more critical for the viability of transformed cells. Hsp90 is absolutely essential for the stabilization/maturation of nuclear hormone receptors, transcription factors, and protein kinases that are commonly misregulated during tumorigenesis [198]. It also serves to buffer the effects of transformation by preventing the aggregation of aberrantly expressed proteins, which would otherwise result in toxic stress signals leading to the progression of programmed cell death [248]. As many of the client proteins of Hsp90 are linked to growth signal pathways, Hsp90 is viewed as key player in the subversion of normal cells toward unrestrained proliferation. Amplifying the corruptive potential of Hsp90 is its ability to facilitate the evolution of neoplastic clones by stabilizing many of the mutated proteins that are often associated with cancerous lesions, including p53, Bcr-Abl, and v-Src [194, 248]. For this reason, Hsp90 is thought to be especially crucial in the development of tumors that result from the inactivation of DNA repair pathways, in which there are extensive pools of variously mutated proteins (Fig. 6.3).

The fact that Hsp90 enhances the catalytic activity of Smyd3, which has been implicated in tumorigenesis [106], further establishes a role for Hsp90 in chaperoning tumorigenesis. However, the mechanism by which Hsp90 enhances Smyd3 methyltransferase activity remains unclear. In their original characterization of Smyd3, Hamamoto et al. mapped the interaction of Smyd3 with Hsp90 to the N-terminus of Smyd3 [106]. It seems reasonable that modest conformational changes in Smyd3, induced by its N-terminal association with Hsp90, may be a mechanism for activating Smyd3. For example, since the N-terminal region of the Smyd3 SET domain (N-SET) is largely responsible for the avidity of cofactor binding [249], conformational alteration of this region may permit a more rapid turnover of S-adenosylmethionine, thereby enhancing the rate of catalysis. Perhaps then, Hsp90 binding alleviates an intrinsic autoinhibition associated with the N-SET of Smyd3. In order to address this question regarding the importance of the Smyd3 N-SET for methyltransferase activity and the potential role of Hsp90 in alleviating autoinhibition by inducing conformational changes in the N-SET of Smyd3, we have designed two N-terminal truncations of Smyd3 to be tested in methyltransferase assays.

The earliest studies highlighting the antitumor capacities of geldanamycin and radicicol credited their abilities to impair the activity of oncogenic protein kinases such as ErbB-2 and v-Src [250]. It was later shown that the biological target of these drugs is actually Hsp90, and that their use blocks the Hsp90-dependent activity of Raf-1, Cdk4, Src-family kinases, and many other ocogenic targets [248, 250]. Since then, immense progress has been made in the

development of pharmacological agents that act as inhibitors of Hsp90. In addition to their role in cancer therapy, these drugs will undoubtedly reveal new insights into the involvement of Hsp90 in diverse physiological processes.

6.5 Conclusion

I conclude that the Hsp90Plus™ system offers an efficient and potentially broad-reaching approach for improving the expression of soluble and active enzymes for research and/or therapeutic purposes. In addition, these studies have provided new insights into the effects of Hsp90 on the catalytic activity of two histone methyltransferases. The prospect that Hsp90 may specifically interact with Smyd3 to alleviate catalytic autoinhibition mediated by the Smyd3 N-SET region is quite novel and it is a priority of our laboratory to validate or disprove this idea.

From its crucial roles in signal transduction to transformation, Hsp90 is a ubiquitous molecular chaperone that influences an expansive array of cellular events through its broad range of protein clientele. Hsp90 has been the focus of intense research for the past 20 years, resulting in the establishment of several overlapping paradigms stemming from the ATP-dependent chaperoning cycle of Hsp90. In spite of this immense progress, many challenges remain. For example, while much is known regarding the ATPase-driven conformational cycling of Hsp90, the precise physical effects imparted by this chaperone that serve to activate its substrates are still poorly understood. In addition, the

currently known repertoire of Hsp90-dependent proteins is far from complete. A more comprehensive listing and characterization of its clients will undoubtedly reveal the vast-reaching governance wielded by Hsp90 as an intermediate custodian of far-reaching physiological processes. As the target for several promising lines of cancer therapeutics, Hsp90 is certain to remain the focus of intense research for many years to come.

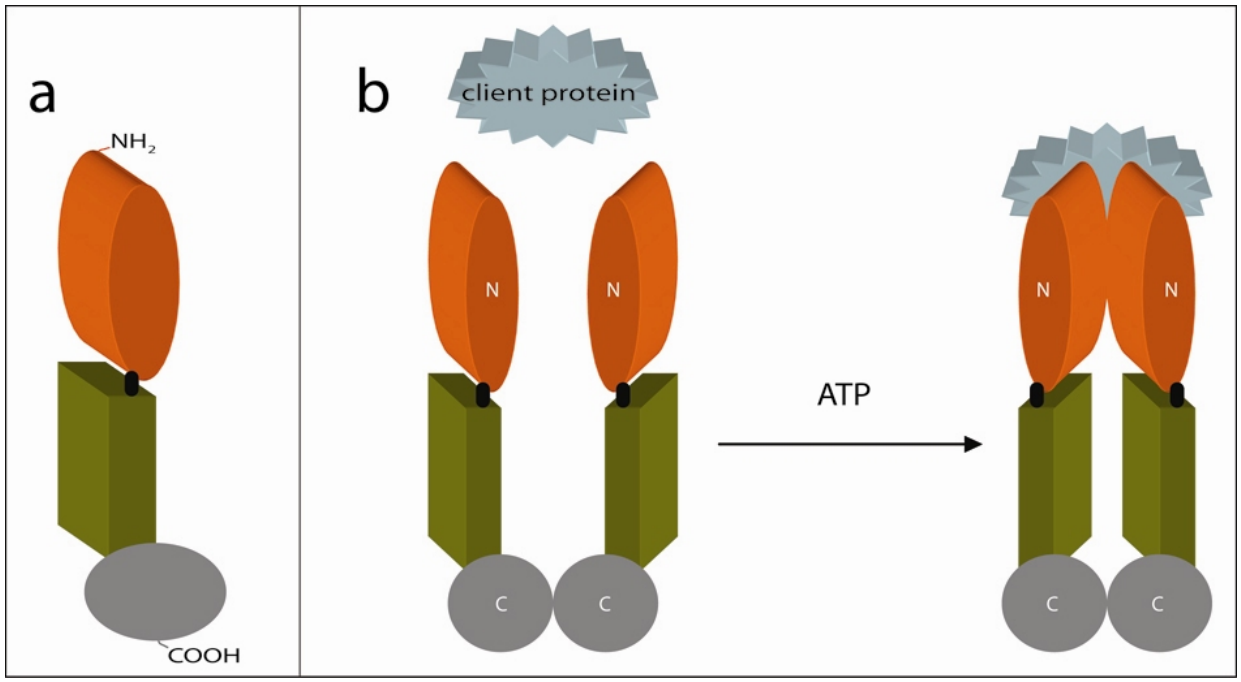

Figure 6.1: The structure of Hsp90 and its ATP-dependent molecular clamp. (a) Schematic representation of Hsp90. The Hsp90 monomer is comprised of three domains: the N-terminal domain responsible for ATP-binding (orange), a core domain (green), and a C-terminal domain that facilitates homodimerization (gray). In eukaryotes, a short charged region links the N-terminal and core domains (black). (b) ATP-driven molecular clamp cycle of Hsp90. In the absence of bound nucleotide, the C-termini (C) of two Hsp90 monomers interact to maintain an antiparallel dimer (left). Concurrently, the N-termini (N) of the Hsp90 homodimer preserve an open-state, facilitating the capture of client proteins (left). On the right, association with ATP induces modest changes in the conformation of Hsp90 that permit a transitory interaction between the opposing N-terminal domains. This produces the closed-form of Hsp90 where clamping of the substrate protein occurs. (Adapted from Brown, et. al [200])

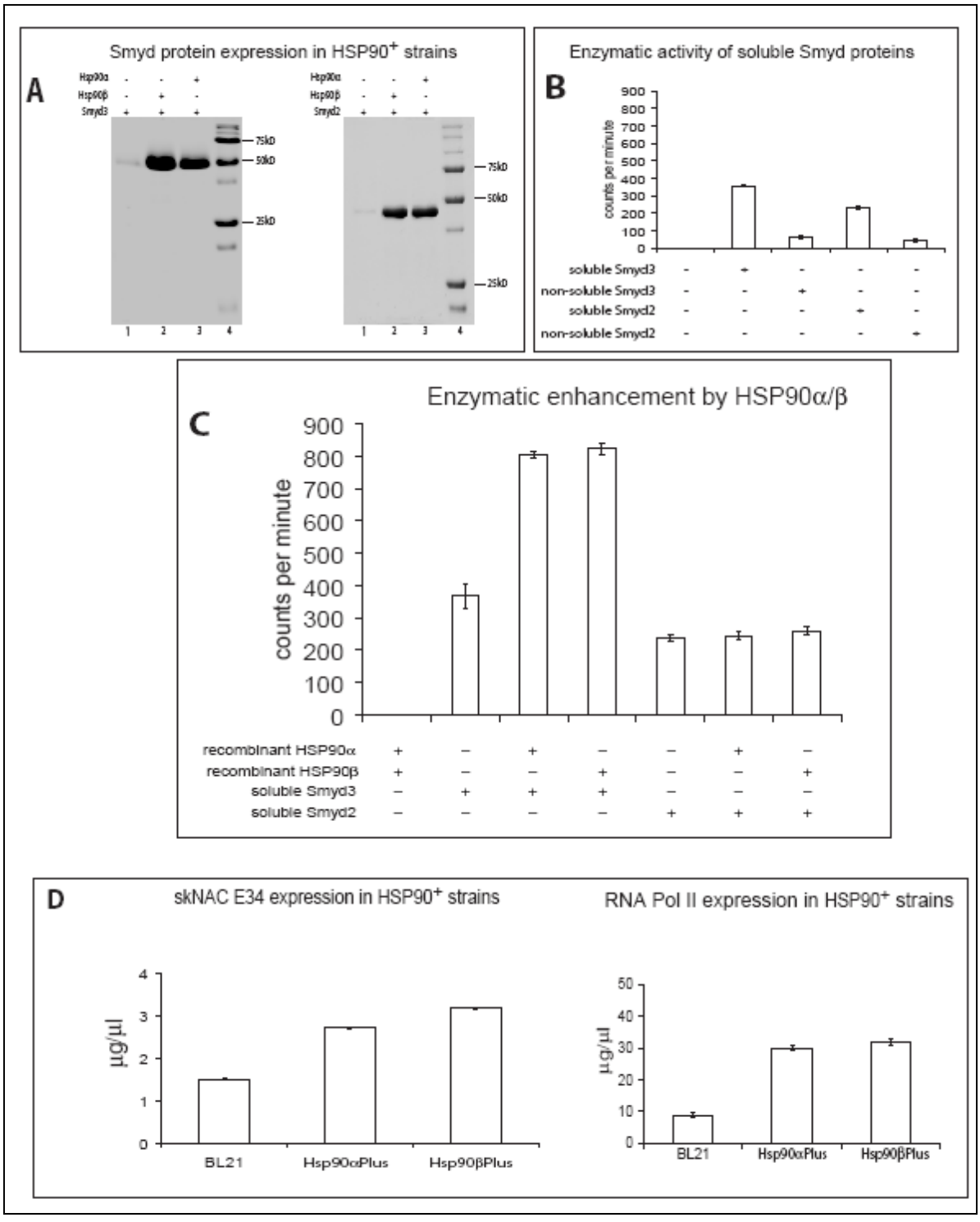

Figure 6.2: Hsp90α and Hsp90β influence solubility and enzymatic activity of Smyd3. (A) Hsp90 isoform-expressing *Escherichia coli* (*E. coli*) strains produce higher yields of soluble Smyd3. Wild type (lane1) and Hsp90 isoform-expressing *Escherichia coli* (*E. coli*) strains, lanes 2 and 3, respectively, were

transformed with a Smyd3 expression construct. Equal amounts (2.5 g wet cell paste) of cultured *E. coli* cells were used for isolation and purification of Smyd3. The migration of molecular weight markers (in kilo Daltons) is indicated. (B) Soluble Smyd3 is enzymatically active. Soluble Smyd3, corresponding to lane 1 of panel (A), was purified from cultured and transformed wild-type *E. coli* cells and subjected to an *in-vitro* histone methyl transferase (HKMT) assay. Non-soluble Smyd3 was purified and re-folded as outlined in chapter 2. Complete reaction mixture without enzyme served as negative control. Incorporation of ^{3}H labeled methyl groups is presented as mean counts per minute from three independent experiments. (C) Hsp90 isoforms enhance the HKMT activity of Smyd3. Soluble Smyd3, corresponding to lane 1 of panel (A), was purified from cultured and transformed wild-type *E. coli* cells. An *in-vitro* HKMT assay was performed using complete reaction buffer plus Hsp90α and Hsp90β, without enzyme, as negative control. The incorporation of ^{3}H labeled methyl groups is presented as mean counts per minute from three independent experiments. (D) Higher yields of soluble skNAC-E34 and RNA Polymerase II C-terminal domain are produced in Hsp90 isoform-expressing *E. coli* strains. Wild type and Hsp90 isoform-expressing *E. coli* strains were transformed with constructs, encoding RNA Polymerase II C-terminal domain and skNAC-E34. Equal amounts (2.5 g wet cell paste) of cultured *E. coli* cells were used for protein purification and subsequent determination of protein concentrations. (Adapted from Brown, et. al [200])

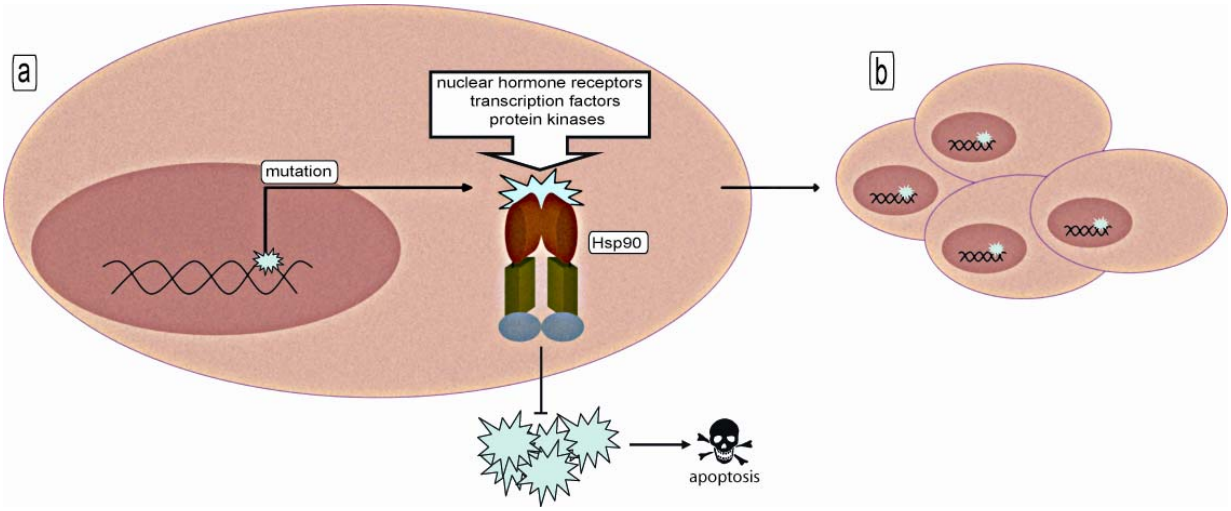

Figure 6.3: Role of Hsp90 in chaperoning tumorigenesis. (a) Hsp90 stabilizes many mutated proteins that mediate cell transformation and prevents the aggregation of aberrantly expressed proteins (blue), which would otherwise result in toxic stress signals leading to the progression of apoptosis. (b) The chaperoning capacity of Hsp90 facilitates the evolution of neoplastic clones. (Adapted from Brown, et. al [200])

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